Mechanisms involved in the pathogenesis of airway hyperresponsiveness in allergic asthma

Mechanisms Involved in the Pathogenesis of Airway Hyperresponsiveness in Allergic Asthma

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This dissertation is dedicated to my father, mother, brother and sister.

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GENERAL INTRODUCTION

Airway hyperresponsiveness (AHR) to a wide variety of pharmacological and/or physical stimuli such as cholinergic agonists, histamine, leukotrienes, prostaglandins, cold air and house dust is one of the most characteristic features of asthmatic airways (Chung & Barnes; 1987). This phenomenon is reflected by bronchoconstriction in asthmatic patients who inhale concentrations of these agents that have no effect in normal subjects. There is a close relationship between the degree of bronchial responsiveness and the severity and frequency of symptoms in asthma (Juniper *et al.*, 1981), so AHR may play a fundamental role in the pathophysiology of asthma. However, the precise mechanisms underlying the AHR remain to be elucidated.

It is well known that the autonomic nervous system regulates airway function, including airway smooth muscle tone, secretions, blood flow, microvascular permeability and the migration and release of inflammatory cells (Barnes, 1986a; 1990a). In addition to classical cholinergic and adrenergic neural pathways, nonadrenergic noncholinergic (NANC) neural mechanisms are also present in the airways. Although neuropeptides may mediate many of the NANC effects in the airways (Barnes, 1992b; Barnes *et al.*, 1991a; 1991b), it has recently been recognized that nitric oxide (NO) may also function as an NANC neurotransmitter (Rand, 1992). There is abundant evidence that neural control of the airways may be abnormal in asthmatic patients, and that neurogenic mechanisms may contribute to the pathophysiology and symptomatology of asthma including AHR (Nadel & Barnes, 1984). Because changes in bronchomotor tone in asthmatic patients occur rapidly, it was suggested many years ago that there might be an abnormality in autonomic neural control of the airways, with an imbalance between excitatory and inhibitory pathways, resulting in excessively reactive airways. Several types of autonomic dysfunction have been proposed in asthma, including enhanced cholinergic, α-adrenergic and nonadrenergic noncholinergic (NANC) bronchoconstrictor mechanisms, or reduced β-adrenergic and NANC bronchodilator effects (Barnes, 1986a). Various abnormalities in airway control have been described in asthma, although is ambiguous whether that these are primary defects or secondary alterations of the disease or by its treatment. Nevertheless, it is likely that neural mechanisms contribute to the symptoms and pathophysiology of asthma.

Because cholinergic nerves are dominant neural bronchoconstrictor pathway in animal and human airways (Barnes, 1986a), there has been considerable interest in whether

cholinergic mechanisms are exaggerated in asthmatic patients. There are several mechanisms that might contribute to cholinergic bronchoconstriction in asthma (Widdicombe et al., 1991). Although there is no direct evidence for an increase in central vagal drive to the airways in asthmatic patients, indirect evidence is provided by an increase in vagal cardiac tone (as determined by the Valsalva manoeuvre and sinus arrhythmia gap) in asthmatic patients, and by an increase in sinus arrhythmia gap at night, which correlates with nocturnal bronchoconstriction (Barnes, 1986a). These observations indicate that the increased vagal tone might be involved in asthma. It is also possible that the reflex bronchoconstriction due to stimulation of sensory receptors in the airways (irritant receptors and C-fiber endings) might be increased by inflammatory mediators. Several mediators, such as histamine, prostaglandins and bradykinin, stimulate sensory receptors in the airways, and it is possible that these receptors may be more easily triggered in asthmatic patients, since airway epithelium may be damaged, or sensory endings may be sensitized by inflammatory mediators such as prostaglandins (Widdicombe *et al.*, 1991). It is reported that bradykinin-induced bronchoconstriction in asthmatic patients is partly reduced by pretreatment with an anticholinergic agent (Barnes, 1992a). More recently, Fox *et al.* (1993) reported that, by using single-fiber recordings in guinea pig airways, bradykinin is very effective in firing C-fibers, whereas other inflammatory mediators have no such effect.

One possible mechanism of the enhanced bronchoconstriction in asthmatic patients is increased release of acetylcholine from parasympathetic nerve endings. There is evidence for facilitated neurotransmission in sensitized animals exposed to allergen (Undem *et al.*, 1991). It has also been thought that inflammatory mediators facilitate acetylcholine release from postganglionic nerve terminals via an effect on prejunctional receptors (Barnes, 1992a). For example, thromboxane, prostaglandin D_2 and tachykinins facilitate acetylcholine release from postganglionic nerves in the airways and thereby exaggerate cholinergic reflex effects (Barnes, 1992a).

At least three types of muscarinic receptors may be differentiated pharmacologically and five distinct receptor subtypes have now been cloned. Muscarinic receptor subtypes have also been recognized in the airways (Barnes, 1990b). Autoradiographic mapping shows that they are differentially distributed in human airways (Mak & Barnes, 1990), and *in situ* hybridization studies show a corresponding differential gene expression (Mak *et al.*, 1992). It has been believed as the following: M_1 receptors in ganglia facilitate ganglionic neurotransmission, M_2 receptors act as feedback inhibitory receptors (autoreceptors) on postganglionic nerve terminals and inhibit acetylcholine release, and M_3 receptors on airway smooth muscle induce bronchoconstriction directly (Barnes, 1990a).

Although adrenergic nerves do not control smooth muscle in human airways directly (Barnes, 1992a), β-agonists have a dramatic effect on relieving asthmatic bronchoconstriction. So, it is logical to suggest that there might be a defect in β-adrenoceptor function in asthmatic patients. From the same reason, it seems probably that circulating catecholamines may play a more important role in regulation of bronchomotor tone (Barnes, 1986c). Although the catecholamines, noradrenaline, adrenaline and dopamine, are present in the circulation, only adrenaline secreted by the adrenal medulla has physiological effects. Some studies have demonstrated that airways from patients who have died of asthma fail to relax normally to isoprenaline, suggesting a defect in β-adrenoceptor function in airway smooth muscle (Goldie et al., 1986; Bai, 1991). On the other hand, α-adrenoceptors which mediate bronchoconstriction have been demonstrated in airways of several species, and may only be demonstrated under certain experimental conditions. There is now, however, considerable doubt about the role of α-adrenoceptors in the regulation of tone in human airways as it has proved difficult to demonstrate their presence functionally or by autoradiography (Spina *et al.*, 1989), and α-blocking drugs do not appear to be effective as bronchodilators.

Recently, it has widely been accepted that the excitatory and inhibitory NANC nervous systems are present in the airways as the third autonomic nervous system. The NANC bronchoconstrictor effect is mediated by sensory neuropeptides, predominantly neurokinin A, as described below. On the other hand, in human airways, bronchodilator nerves that are non-adrenergic in nature have also been demonstrated both *in vitro* using electrical field stimulation and *in vivo* using reflex activation with chemical or mechanical irritation (Lammers *et al.*, 1992). A defect in the NANC bronchodilator nerves has been proposed in asthma, as this is the only neural bronchodilator pathway in human airways. It has been reported that a defect in NANC bronchodilatation is observed after antigen exposure in cats (Miura et al., 1992). However, in patients with mild asthma, no reduction in the NANC bronchodilator reflex is apparent (Lammers et al., 1989). Vasoactive intestinal polypeptide and nitric oxide are thought as the neurotransmitters of bronchodilator NANC nerves in the airways (Barnes, 1991a; Rand, 1992).

One of the most important characteristics in asthmatic patients is severe airway inflammation. There has been increasing interest in the role played by the inflammatory changes present in the airways of asthmatic patients in the development and maintenance of AHR. The airways of patients died by acute asthma attack show several characteristics of a severe inflammatory response. The violent submucosal swelling, that contributes in part to the airway obstruction in asthma, is due to congested blood vessels, plasma exudation and inflammatory cells infiltration consisting predominantly of eosinophils (Dunnill, 1960;

Houston *et al.*, 1953). In addition, there is marked epithelial cell loss as shown by the denudation of the airway surface and by clumps of epithelial cells in sputum, and there is also an increased mucus secretion (Dunnill, 1960; Houston *et al.*, 1953). No detailed physiological examination of the airways of stable asthmatic patients is available but bronchoalveolar lavage of these patients reveals a larger number of eosinophils and, to a lesser extent, neutrophils within the airway lumen when compared to normal subjects (Godard *et al.*, 1982; Flint *et al.*, 1985). Biopsies of the airway mucosa of stable asthmatics have confirmed the presence of epithelial cell damage, particularly of the ciliated cell type (Laitinen et al., 1985) and the submucosal infiltration of eosinophils (Glynn & Michaels, 1960). Thus, regardless of the extent of asthmatic symptoms, it seems likely that airway inflammation is a common feature of bronchial asthma.

The immediate type of bronchoconstriction observed in allergic bronchial asthmatics has been thought to occur as a result of the antigen-antibody reaction on airway luminal surface and/or submucosa. So, it has been proposed that the mast cell is the initiator of inflammatory responses to allergic stimuli in the airways (Casale & Kaliner, 1984). Although mast cells are abundant in lung tissue (Guerzon *et al.*, 1979), they only form a relatively small proportion of the cells recovered from the airway lumina of normal and asthmatic subjects (Flint *et al.*, 1985). It has been suggested that activation and degranulation of these relatively few mast cells lead to increased mucosal permeability with subsequent activation of tissue mast cells (Hogg, 1981). The mast cells can release a wide variety of inflammatory mediators that can mimic some of the features of bronchial asthma including smooth muscle contraction and mucous secretion (Casale & Kaliner, 1984). It also generates chemotactic factors for inflammatory cells (*e.g.*, eosinophils, neutrophils), such as hydroxyeicosatetraenoic acids (HETE's) and leukotriene B_4 (Robinson & Holgate, 1985; Wasserman, 1983). On the other hand, it has been reported that 15-HETE, generated from eosinophils (Turk *et al.*, 1982) and airway epithelial cells (Hunter *et al.*, 1985), and major basic protein released from eosinophils can activate mediator release from mast cells (Goetzl *et al.*, 1983; O'Donell *et al.*, 1981). It seems that the cell-cell interactions are highly complex.

Alveolar macrophages are in greater abundance within the lumen of the airways than mast cells, and also generate chemotactic factors for inflammatory cells (Gosset *et al.*, 1984; Hunninghake *et al.*, 1978). This phenomenon is thought to occur through the activation of their low-affinity surface IgE receptors by antigen (Joseph *et al.*, 1983). It was also reported that alveolar macrophages can release the putative inflammatory mediator, platelet-activating factor (PAF) (Arnoux *et al.*, 1980; 1983).

It is generally accepted that eosinophils have a pro-inflammatory role. The infiltrating

eosinophil can generate mediators that could be involved in enhancing bronchial responsiveness. It is reported that eosinophilic cationic protein and major basic protein, both major components of eosinophilic granules (Ackerman *et al.*, 1983), are cytotoxic to the respiratory epithelium (Gleich *et al.*, 1979) and could therefore account for the denudation of the epithelium observed in asthmatic patients (Filley *et al.*, 1982). Eosinophils were also reported to have the capacity to generate leukotriene C_4 (Weller *et al.*, 1983) and PAF (Lee *et al.*, 1984).

The neutrophil is an extremely potent cell capable of generating prostaglandins, thromboxane, leukotrienes and PAF, despite its relative paucity in the airway wall of asthmatic patients (Chung & Barnes, 1987). However, few studies reported the implication of neutrophils in allergic asthmatic patients.

Recently, PAF has been considered as one of the most important mediators which induce AHR (Chung & Barnes, 1987). PAF causes a long-lasting, intense inflammatory reaction in the airways with the participation of neutrophils, macrophages, epithelial cells and platelets (Camussi *et al.*, 1983) and increased microvascular permeability and oedema (Humphrey *et al.*, 1982; Heffner *et al.*, 1983). In addition to its capacity to reproduce these pathological features that are characteristic of the asthmatic airways, PAF is a potent bronchoconstrictor agent in human *in vivo* and induces AHR (Cuss *et al.*, 1986). Evidence for PAF release has been obtained following antigenic challenge in asthmatic patients by the demonstration of a selective reduction of the aggregatory response of circulating platelets to PAF (Thompson *et al.*, 1984). It has also been reported that increased levels of circulating PAF were detected in asthmatic patients (Grandel *et al.*, 1983). The exact mechanisms by which PAF causes AHR remain to be determined. It has been suggested that PAF may mediate the epithelial damage seen in the asthmatic airways through the recruitment and stimulation of eosinophils. This epithelial damage may result in the exposure of C-fiber endings which may then be stimulated by various mediators released during the inflammatory process (Coleridge & Coleridge, 1984). For instance, bradykinin acts on C-fiber endings to cause the release of sensory neuropeptides such as substance P (SP) and neurokinin A (NKA) (Barnes, 1986b). This local reflex may serve to enhance inflammatory responses seen in the asthmatic airways with mucous hypersecretion and bronchial oedema through these neuropeptides. In addition, it has also been reported that these neuropeptides potentiate mediator release and inflammatory cell infiltration (Payan *et al.*, 1984). On the other hand, it was also reported that PAF antagonists had no effect on the anaphylactic bronchoconstriction although these antagonists inhibited the PAF-induced bronchoconstriction in guinea pigs (Danko *et al.*, 1988). These findings suggest that PAF would not be a final and/or important mediator involved in the asthmatic reaction

and/or the pathogenesis of the AHR, although PAF has a potency to cause bronchoconstriction (Danko *et al.*, 1988; Misawa & Takata, 1988) and AHR (Chung *et al.*, 1986b).

More recently, the contribution of the neurogenic inflammation in the airways in asthmatic patients has been noticed. Unmyelinated sensory nerves (C-fibers) in the airways contain several neuropeptides, such as SP, NKA and calcitonin gene-related peptide (CGRP), which may be released from sensory nerves on antidromical activation of the nerve via an axon reflex mechanism. Release of neuropeptide from sensory nerves in the airways might result in inflammation of the airways. This is readily demonstrated in some species but whether it is relevant in human airways is not yet certain. In a chronic animal model of antigen-induced AHR, depletion of sensory neuropeptides by prior systemic treatment with capsaicin, the pungent agent of hot peppers, results in the abolition of the hyperresponsiveness (Matsuse *et al.*, 1991), whereas, in acute models of allergen exposure, capsaicin pretreatment has no effect (Lö-tvall *et al.*, 1991). This suggests that sensory neuropeptides may become more important in chronic inflammation.

It has been reported that SP-immunoreactive nerves are increased in the airways of patients who died of asthma (Ollerenshaw *et al.*, 1991). This could be the result of chronic inflammation resulting in proliferation of sensory nerves in the airways. Inflammatory signals may stimulate increased synthesis of neuropeptides in sensory neurons.

The denudation of airway epithelium in asthmatic airways may expose sensory nerve endings, and these may be triggered or sensitized by certain inflammatory mediators, such as prostaglandins. Asthma may therefore be associated with hyperaesthesia of the airways. For instance, it is likely that cough and chest tightness, common symptoms of asthma, are a reflection of this airway hyperaesthesia. Bradykinin is thought to be particularly important in activating sensitized sensory nerves and, when inhaled to asthmatic patients, it induces marked dyspnoea and coughing, which are reminiscent of an asthma attack (Barnes, 1992a). Bradykinin is formed from plasma which exudes into the asthmatic airway lumen as a result of microvascular leakage. Activation of these sensory nerves may then cause antidromic stimulation of branches of the nerves, with release of their neuropeptides via an axon reflex (Barnes, 1992a).

SP and NKA belong to the tachykinin family and activate different subtypes of tachykinin receptor. NKA is a potent constrictor of human bronchi *in vitro* (Solway & Leff, 1991) and is considerably more potent than SP, indicating that an NK_2-receptor is present on airway smooth muscle. SP is a potent inducer of microvascular leakage in the airways and is more potent than NKA in this respect. SP also stimulates mucous secretion more potently both in animal and human bronchi *in vitro*. It is also reported that SP is by far the most potent of

these peptides in stimulating goblet cell secretion (Kuo *et al.*, 1990). This may be important in asthma, as goblet cells are the only source of mucous glycoproteins in peripheral airways. All of these inflammatory responses are thought to be mediated by NK_1-receptors. It is reported that Northern analysis of asthmatic lungs indicates an increased amount of NK_1-receptor mRNA, which may result from the effects of various cytokines on NK_1-receptor gene transcription (Peters *et al.*, 1992).

These sensory neuropeptides are degraded predominantly by neutral metalloendopeptidase (NEP, EC 3.4.24.11, enkephalinase) which is localized mainly in airway epithelium. An inhaled NEP inhibitor, thiorphan, potentiates the bronchoconstrictor effect of inhaled NKA in normal human subjects, indicating the presence of NEP on the surface of human airways (Cheung *et al.*, 1992), and NEP has been localized immunocytochemically and its gene expression determined using *in situ* hybridization in human airways (Baraniuk *et al.*, 1991). However, Nichol *et al.* (1992) reported that another NEP inhibitor, acetorphan, does not cause bronchoconstriction in asthmatic patients, indicating that there may not be increased basal release of neuropeptides in asthmatic airways. Whether NEP activity is reduced in asthmatic airways is not yet certain, and this is an interesting issue including the role of sensory neuropeptides on the pathogenesis of AHR.

To understand the underlying mechanisms of AHR in allergic asthmatic patients, it is very important to develop an animal model of AHR which closely resembles that observed in asthmatic patients. Therefore, in my present experiment, at first, I tried to develop an animal model of AHR by using sensitized rats. Some of the mechanisms of the pathophysiology of AHR cleared by using this animal model of AHR will be introduced and discussed in this thesis.

AIM & SCOPE

The specific aims of the proposed research are:

In Chapter 1;

To understand the underlying mechanisms of AHR in allergic asthma, I tried to develop an animal model of AHR by using actively sensitized rats. The correlation between AHR and airway inflammation was examined by using this antigen-induced AHR model. Whether AHR is also obtained at the *in vitro* bronchial level was then examined.

In Chapter 2;

Strain differences in the pathogenesis of antigen-induced AHR were examined by using Brown-Norway (a high IgE responder), Long-Evans Cinnamon (which has dysfunction of helper T cell) and Wistar rats (as a normal responder).

In Chapter 3;

The effects of ozagrel (a thromboxane A_2 synthase inhibitor) and CV-3988 (a PAF receptor antagonist) on the pathogenesis of antigen-induced AHR in rats were examined.

In Chapter 4;

The effects of sensory neuropeptides depletion on the pathogenesis of antigen-induced AHR in rats were examined. The effect of repeated antigenic challenge to sensitized rats on the airway tissue NEP activity was also examined.

In Chapter 5;

The effect of NEP inhibitors on the airway responsiveness to acetylcholine in nonsensitized normal rats was examined. The mechanism of the increased airway responsiveness to acetylcholine by neuropeptides was also investigated.

In Chapter 6;

The origin of Ca^{2+} contributing to the enhancement of acetylcholine-induced bronchial smooth muscle constriction in airway hyperresponsiveness induced by antigen challenge was investigated.

CHAPTER 1:
Establishment of an animal model of airway hyperresponsiveness

Introduction

Nonspecific airway hyperresponsiveness (AHR) is a common feature in individuals with allergic bronchial asthma (Cockcroft *et al.*, 1977; Magnussen & Nowak, 1989). In allergic asthma, inhalation of allergen causes not only an immediate and reversible bronchoconstriction but also AHR to stimuli such as histamine and acetylcholine (ACh). The importance of the increased responsiveness in the pathogenesis of asthma has been suggested by its correlation with the severity of this disease (Cockcroft *et al.*, 1977; Hargreave *et al.*, 1981). It is thus very important to understand the underlying mechanisms of AHR for the sake of asthma therapy.

To understand the mechanisms of AHR, various animal models have been developed. O' Byrne *et al.* reported that prostaglandin $F_{2\alpha}$ (O' Byrne *et al.*, 1984), leukotriene B_4 (O' Byrne *et al.*, 1985) and ozone (Aizawa *et al.*, 1985) induced AHR to ACh in normal dogs. However, it is unlikely that these dog models resemble human allergic asthma because these animals were not made allergic. A frequently used animal model is the model using guinea pigs that are sensitized with ovalbumin (OA) together with some adjuvant (Ishida *et al.*, 1989; Boichot *et al.*, 1991). However, this species has been considered to be immunologically different from humans with regard to the mechanisms of allergic asthma involving antibody (Wanner *et al.*, 1990).

Because allergic bronchoconstriction in rats has certain features in common with human allergic asthma, rats show good potential as an allergic asthmatic animal model (Elwood *et al.*, 1991; Renzi *et al.*, 1992). It has been demonstrated that the immunoglobulin E (IgE) antibody was easily generated when rats were sensitized with DNP-*Ascaris* antigen (Tada & Okumura, 1971). The sensitized rats also exhibit early and late asthmatic responses to antigen inhalation (Eidelman *et al.*, 1988). In the present study, to investigate the underlying mechanisms of AHR, we first tried to develop a new animal model of AHR by utilizing this species. The relationship between AHR and airway inflammation in our rat model was also examined.

Experiment 1-1: Effect of single antigenic challenge on *in vivo* airway responsiveness in actively sensitized rats.

Materials and methods

Antigen challenge

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were housed under standard laboratory conditions with free access to food and water. Animals were sensitized with 2,4-dinirophenylated *Ascaris suum* extract (DNP-Asc, 2 mg protein, *s.c.*) together with killed *Bordetella pertussis* ($2x10^{10}$) as an adjuvant and were boosted by DNP-Asc (0.5 mg protein, *i.m.*) 5 days later, according to the method of Tada and Okumura (1971). Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc (6 mg protein/ml, 5-6 ml) with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo) for 20 min under conscious state in a plexiglass box (300x200 mm, height: 150 mm). These animals were designated as the single challenge group and used for experiments 24 hr after the antigenic challenge. Control rats received the same immunization, but inhaled saline instead of the DNP-Asc challenge dose.

In vivo determination of airway responsiveness

The determination of airway responsiveness to ACh or neurokinin A (NKA) was measured 24hr after the antigenic challenge. Rats were anesthetized with urethane (2 g/kg, *i.p.*), placed in the supine position and ventilated artificially through a tracheal cannula at a frequency of 70 breaths/min. Respiratory volume was adjusted at the beginning of the experiment so that ventilation overflow (VO) was 0.5 ml in each rat. Spontaneous respiration was stopped by an injection of pancuronium bromide (1 mg/kg, *i.v.*). The bronchomotor tone was measured by the modified Konzett-Rössler method (Konzett & Rössler, 1940) (Fig. 1-1). The lung was inflated at a fixed volume of air under a constant pressure (5 cmH_2O), and VO was continuously recorded with a combination of a pneumotachograph (TP-602T, Nihon Kohden) and an integrator (EI-601G, Nihon Kohden). Systemic blood pressure was monitored with a pressure transducer (DX-300, Nihon Kohden) from a cannula inserted into the left carotid artery, and heart rate was measured with a tachometer (AT-601G, Nihon Kohden) with the systemic blood pressure as the trigger. All the above parameters were recorded on a polygraph (RM-6000, Nihon Kohden).

The rats were subjected to cumulative inhalation of ACh (0.001-0.03 %) or NKA

(0.0001-0.01 %), each for 3 min, by aerosolizing the solution contained in a specially devised plastic cylindrical chamber (capacity: 25 ml) that was introduced in an ultrasonic nebulizer (TUR-3200, Nihon Kohden). The ultrasonic nebulizer with the plastic chamber was placed into the respiratory circuit so that the aerosolized mist was inhaled into the airway each time of ventilation.

Drugs

The following drugs were used: acetylcholine chloride (purchased from Daiichi Pharmaceutical Co., Ltd., Tokyo); NKA (from Peptide Institute, Inc., Osaka); pancuronium bromide (from Sankyo Pharmaceutical Co., Ltd., Tokyo) and urethane (ethyl carbamate; from Sigma Chemical Co., Ltd., St. Louis, USA). Heparin sodium was from Kodama Co., Ltd. (Tokyo).

For the *in vivo* studies, ACh, NKA and pancuronium bromide were freshly made just before use. Urethane was dissolved in saline and stocked at room temperature. Heparin solution was diluted freshly in saline each day.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test. The potency ratio of the *in vivo* bronchoconstrictor response to ACh or NKA was calculated by parallel line assay.

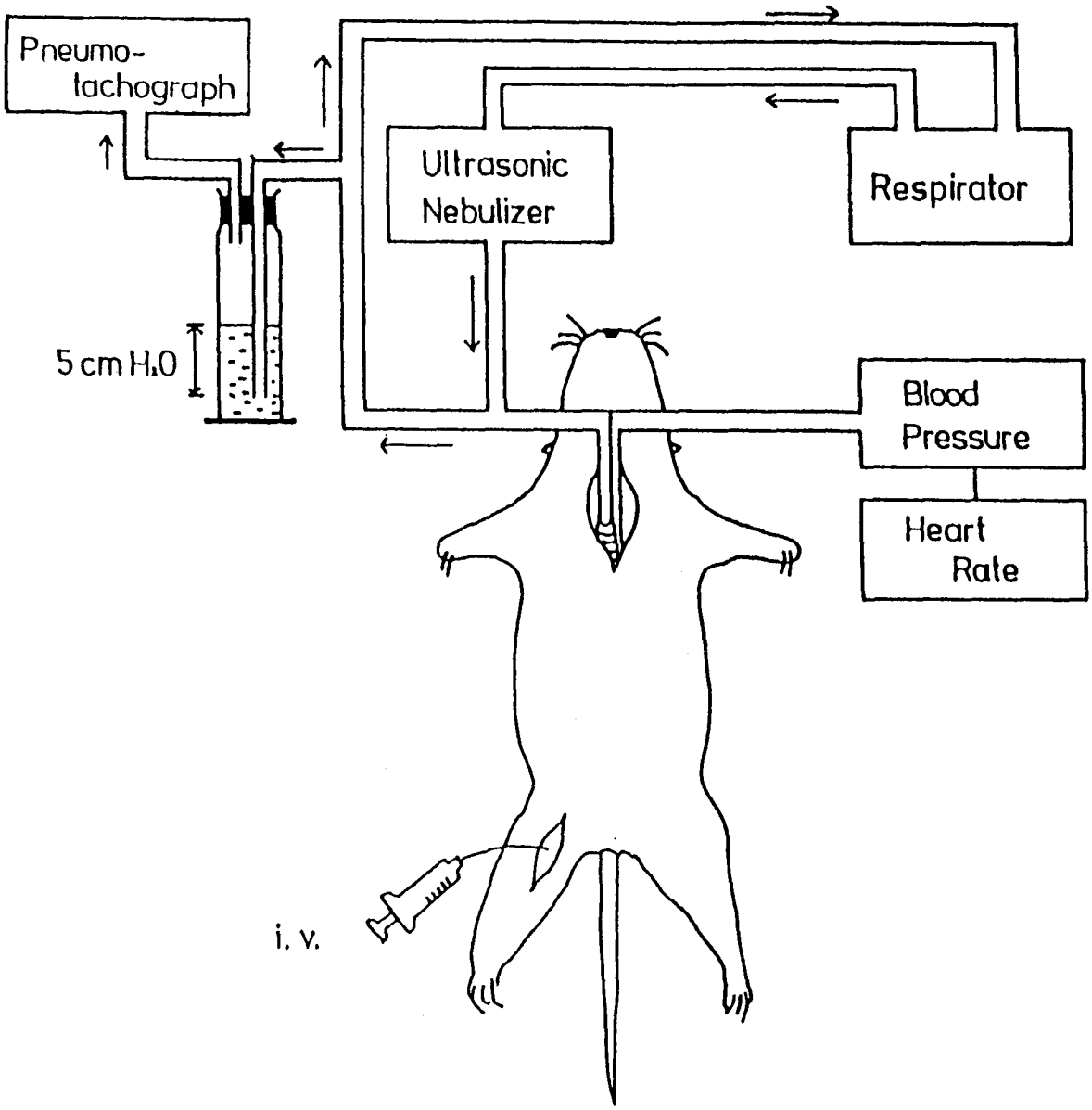


Figure 1-1

Scheme of the in vivo experimental method. Acetylcholine was inhaled with an ultrasonic nebulizer to determine airway responsiveness in anesthetized rats.

Results

Inhalation of four cumulative aerosols of ACh (0.001, 0.003, 0.01 and 0.03 %, each for 3 min) to anesthetized control rats induced a concentration-related bronchoconstriction (Fig. 1-2). When the animals were challenged only once with DNP-Asc, the concentration-response curve to ACh was shifted to the left 3.2-fold in terms of the concentration ratio, 24 hr after the challenge. The airway responsiveness to ACh was significantly enhanced at concentrations of 0.001 and 0.003 % ($p<0.05$). Similar results were obtained with NKA (0.0001, 0.0003, 0.001, 0.003 and 0.01 %, each for 3 min) (Fig. 1-3); the concentration-response curve was shifted to the left (sixfold) 24 hr after the single antigenic challenge. The airway responsiveness to NKA was significantly enhanced at concentrations of 0.0003 and 0.001 % ($p<0.05$).

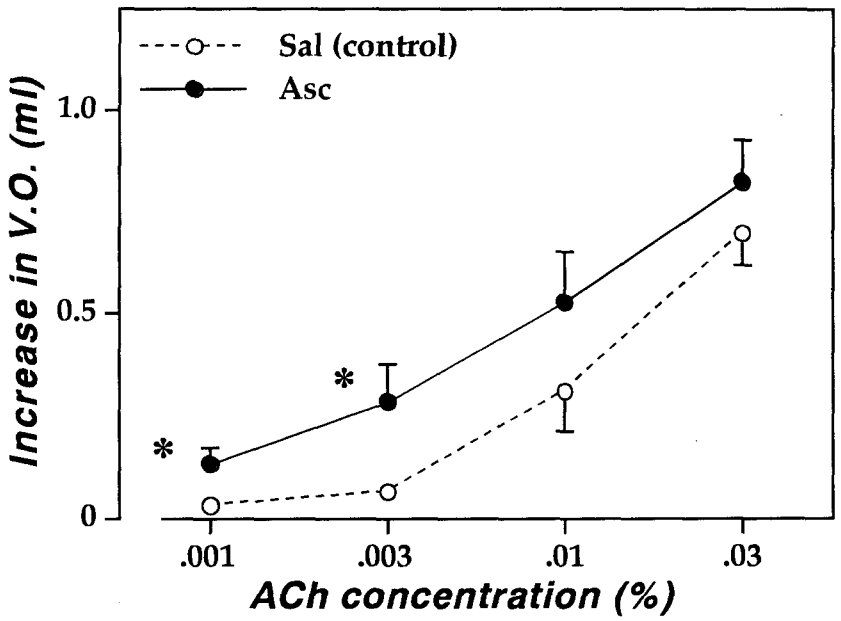


Figure 1-2

The concentration-response curves of bronchomotor response to aerosolized acetylcholine (ACh) after single challenge with DNP-*Ascaris* antigen (Asc) in anesthetized rats. Each point represents the mean with S.E. from 5 (saline control: Sal) and 6 (Asc) experiments. V.O.: ventilation overflow as an index of bronchoconstriction (see **Materials and methods**). *$p<0.05$ vs. control group.

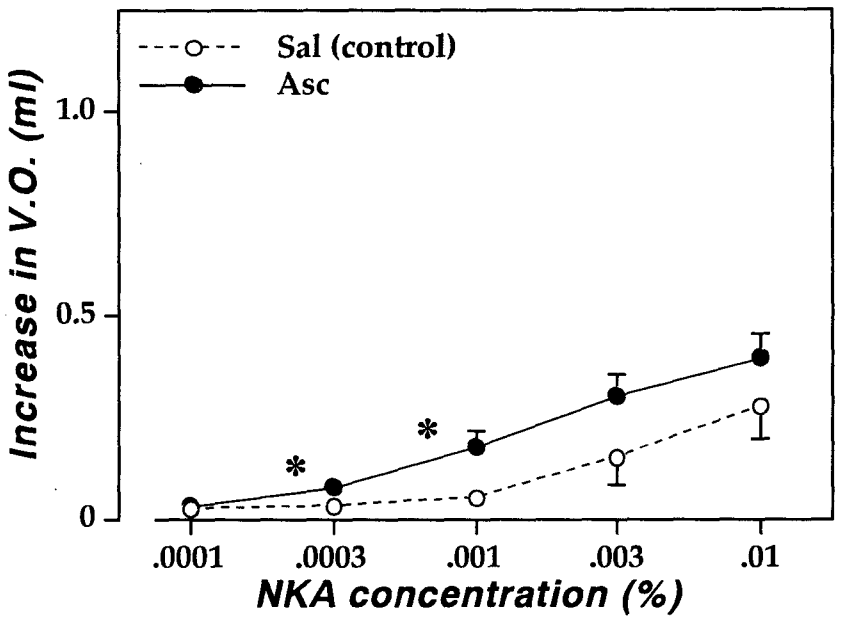


Figure 1-3

The concentration-response curves of bronchomotor response to aerosolized neurokinin A (NKA) after single challenge with DNP-*Ascaris* antigen (Asc) in anesthetized rats. Each point represents the mean with S.E. from 5 (saline control: Sal) and 6 (Asc) experiments. V.O.: ventilation overflow as an index of bronchoconstriction (see **Materials and methods**). *$p<0.05$ vs. control group.

Experiment 1-2: Effect of repeated antigenic challenge on *in vivo* airway responsiveness in actively sensitized rats.

Materials and methods

Antigen challenge

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were used. Animals were sensitized and boosted with DNP-Asc as described in **Experiment 1-1**. Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc (6 mg protein/ml, 5-6 ml) with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo) for 20 min under conscious state in a plexiglass box (300x200 mm, height: 150 mm). Then the animals received 3 times repeatedly antigenic challenge every 48hr by the same challenge method as described above. These animals were used for experiments 24 hr after the last antigenic challenge. Control rats received the same immunization, but inhaled saline instead of the DNP-Asc challenge dose.

In vivo determination of airway responsiveness

The determination of airway responsiveness to ACh or neurokinin A (NKA) was measured 24hr after the last antigenic challenge by the method described in **Experiment 1-1**.

Drugs

The following drugs were used: acetylcholine chloride (purchased from Daiichi Pharmaceutical Co., Ltd., Tokyo); NKA (from Peptide Institute, Inc., Osaka); pancuronium bromide (from Sankyo Pharmaceutical Co., Ltd., Tokyo) and urethane (ethyl carbamate; from Sigma Chemical Co., Ltd., St. Louis, USA). Heparin sodium was from Kodama Co., Ltd. (Tokyo).

For the *in vivo* studies, ACh, NKA and pancuronium bromide were freshly made just before use. Urethane was dissolved in saline and stocked at room temperature. Heparin solution was diluted freshly in saline each day.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test. The potency ratio of the *in vivo* bronchoconstrictor response to ACh or NKA was calculated by parallel line assay.

Results

The airway responsiveness to inhaled ACh was much more markedly increased 24 hr after the repeated antigenic challenge.

Figure 1-4 shows the concentration-response curve for inhaling ACh 24 hr after the last antigenic challenge. In the repeated challenge group, a dramatic leftward shift of the concentration-response curve (12.0 times; its 95 % confidence interval: 6.1-63.9, $p<0.05$) was observed, as compared with the single challenge group. Similar results were obtained with cumulative inhalation of NKA; the concentration-response curve was shifted to the left (12.4-fold; its 95 % confidence interval: 9.0-19.5, $p<0.05$) 24 hr after the last antigenic challenge (Fig. 1-5).

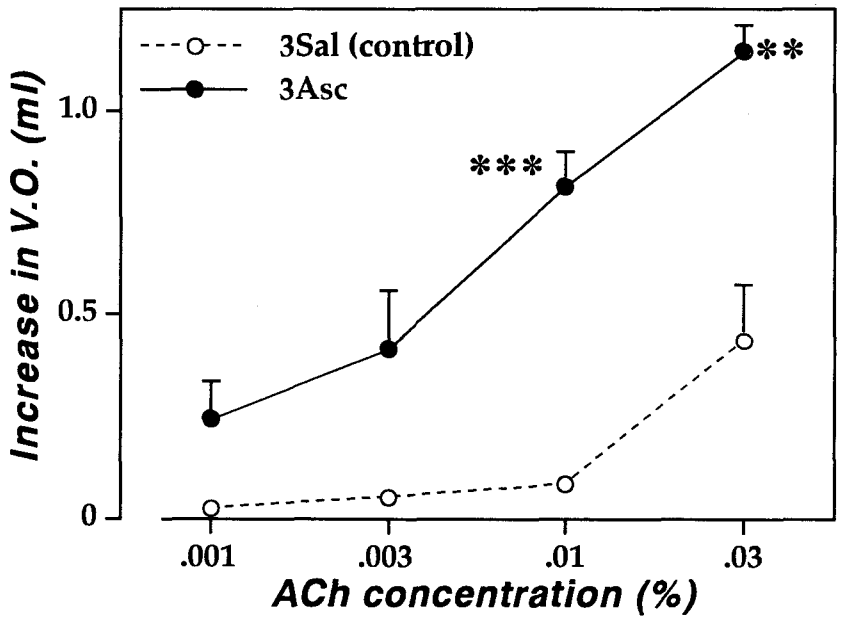


Figure 1-4

The concentration-response curves of bronchomotor response to aerosolized acetylcholine (ACh) after repeated challenge with DNP-*Ascaris* antigen (Asc) in anesthetized rats. Each point represents the mean with S.E. from 6 (saline control: 3Sal) and 7 (3Asc) experiments. V.O.: ventilation overflow as an index of bronchoconstriction (see **Materials and methods**). **p<0.01 and ***p<0.001 vs. control group.

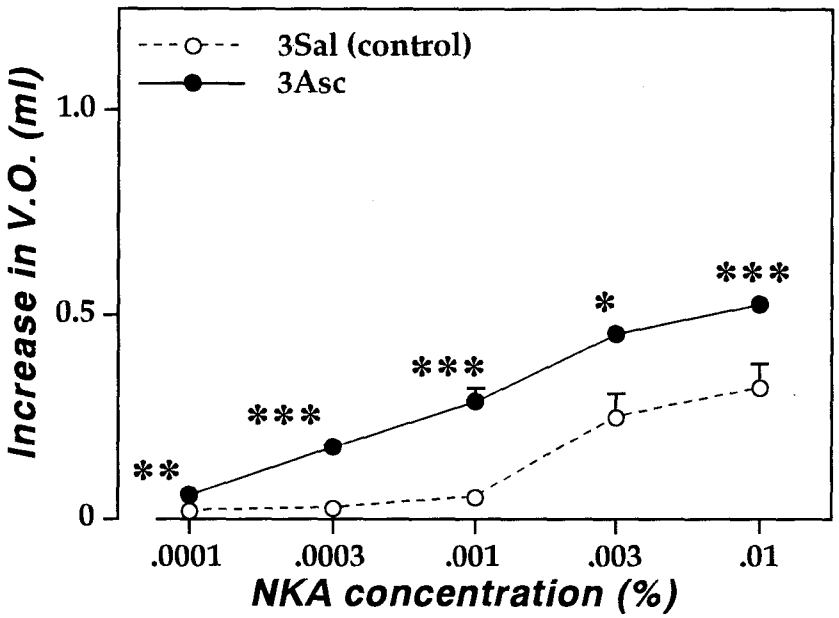


Figure 1-5

The concentration-response curves of bronchomotor response to aerosolized neurokinin A (NKA) after repeated challenge with DNP-*Ascaris* antigen (Asc) in anesthetized rats. Each point represents the mean with S.E. from 5 (saline control: 3Sal) and 8 (3Asc) experiments. V.O.: ventilation overflow as an index of bronchoconstriction (see **Materials and methods**). *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

Experiment 1-3: Comparison of antigen induced-bronchoconstriction between single and repeated antigen challenged rats.

Materials and methods

Animals

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were used. Animals were sensitized and boosted with DNP-Asc as described in **Experiment 1-1**.

In vivo determination of DNP-Asc-induced bronchoconstriction

At 8 days after the first immunization in the single challenge group and at the third challenge in the repeated challenge group, the antigenic challenge was performed under anesthesia by inhaling DNP-Asc for 20 min through the tracheal cannula, and the DNP-Asc-induced immediate type of bronchoconstriction was measured by the modified Konzett-Rössler method (Konzett & Rössler, 1940) described in **Experiment 1-1** and compared between the two groups.

Drugs

The following drugs were used: pancuronium bromide (purchased from Sankyo Pharmaceutical Co., Ltd., Tokyo), urethane (ethyl carbamate; from Sigma Chemical Co., Ltd., St. Louis, USA) and heparin sodium (from Kodama Co., Ltd., Tokyo).

Pancuronium bromide were freshly made just before use. Urethane was dissolved in saline and stocked at room temperature. Heparin solution was diluted freshly in saline each day.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

Results

Eight days after the first immunization, inhalation of aerosolized DNP-Asc (6 mg protein/ml, 20 min) to sensitized rats under anesthesia (First challenge) induced an increase in ventilation overflow (VO as an index of bronchoconstriction) (Table 1). A similar, but slightly stronger, response was confirmed at the last antigenic challenge in the repeated challenge

group (Last challenge). However, there was no significant difference between the groups in the maximal increase in VO (VO_{max}) nor the time for reaching VO_{max} (T_{max}) during inhalation of DNP-Asc.

Table 1-1. The maximal increase in ventilation overflow (ΔVO_{max}) and the time for reaching VO_{max} (T_{max}) during DNP-Asc (6 mg protein/ml) challenge for 20 min under anesthesia

	n	ΔVO_{max} (ml)	T_{max} (min)
First challenge	**6**	**0.41±0.05**	**15.1±1.8**
Last challenge	**5**	**0.51±0.05^{ns}**	**13.8±1.6^{ns}**

ΔVO_{max} and T_{max} were expressed as the mean±S.E. First challenge: 8 days after the first immunization (matched for the antigenic challenge of the single challenge group). Last challenge: 12 days after the first immunization (matched for the last antigenic challenge of the repeated challenge group). ^{ns}: no significant difference from the first challenge group.

Experiment 1-4: Time course of *in vivo* airway hyperresponsiveness to acetylcholine after repeated antigenic challenge.

Materials and methods

Antigen challenge

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were used. Animals were sensitized, boosted and repeatedly challenged with DNP-Asc as described in **Experiment 1-2**. Control rats received the same immunization, but inhaled saline instead of the DNP-Asc challenge dose.

In vivo determination of airway responsiveness

The determination of airway responsiveness to ACh was measured 12, 24, 48 or 72 hr after the last antigenic challenge by the method described in **Experiment 1-1**.

Passive cutaneous anaphylaxis (PCA)

To measure PCA titer, the blood specimens were obtained from the heart at 8 and 12 days after the first immunization and at 12, 24, 48 and 72 hr after the last antigenic challenge. The blood specimens were immediately ice-cooled for 10 min and then centrifuged (3000 rpm, 4 °C for 10 min). The sera obtained were stored at -35 °C until used.

Serial dilutions (4-512) of the serum obtained from individual bleedings were injected intradermally in 0.05 ml quantities into the shaved backs of normal rats. After 48 hr, the rats were *i.v.*-injected with DNP-Asc (2 mg protein/200 g body weight) into the tail veins. After 30 min, they were exsanguinated from the cervical aortae, and the skin was reflected for measurement of the extent of extravasation of the dye. When the diameter was ≥5 mm, the dilution was determined to be end point.

Drugs

The following drugs were used: acetylcholine chloride (purchased from Daiichi Pharmaceutical Co., Ltd., Tokyo); pancuronium bromide (from Sankyo Pharmaceutical Co., Ltd., Tokyo); Evans blue (from Merck Co., Ltd., New Jersey, USA) and urethane (ethyl carbamate; from Sigma Chemical Co., Ltd., St. Louis, USA). Heparin sodium was from Kodama Co., Ltd. (Tokyo).

ACh and pancuronium bromide were freshly made just before use. Evans blue and urethane were dissolved in saline and stocked at room temperature. Heparin solution was

diluted freshly in saline each day.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test. The PCA titer of each group is expressed as the mean and its statistical significance was determined by the Wilcoxon rank-sum test.

Results

Figure 1-6 shows the time course of airway responsiveness to ACh after the last DNP-Asc challenge in the repeated challenge group. The airway responsiveness to ACh was stronger in the following order, 24 hr>12 hr>48 hr>72 hr after the last DNP-Asc challenge in the repeated challenge group. The most severe airway hyperresponsiveness was thus shown at 24 hr after the last antigenic challenge. At 72 hr after the last antigenic challenge, however, the airway responsiveness almost recovered to the control level.

The antibody titer was measured by 48-hr passive cutaneous anaphylaxis (PCA) at 8 and 12 days after the first immunization and at 12, 24, 48 and 72 hr after the last antigenic challenge (Table 1-2). There was no significant difference in PCA titers among the groups, except that the titer at 72 hr after the last antigenic challenge was significantly lower than that at 24 hr ($p<0.05$).

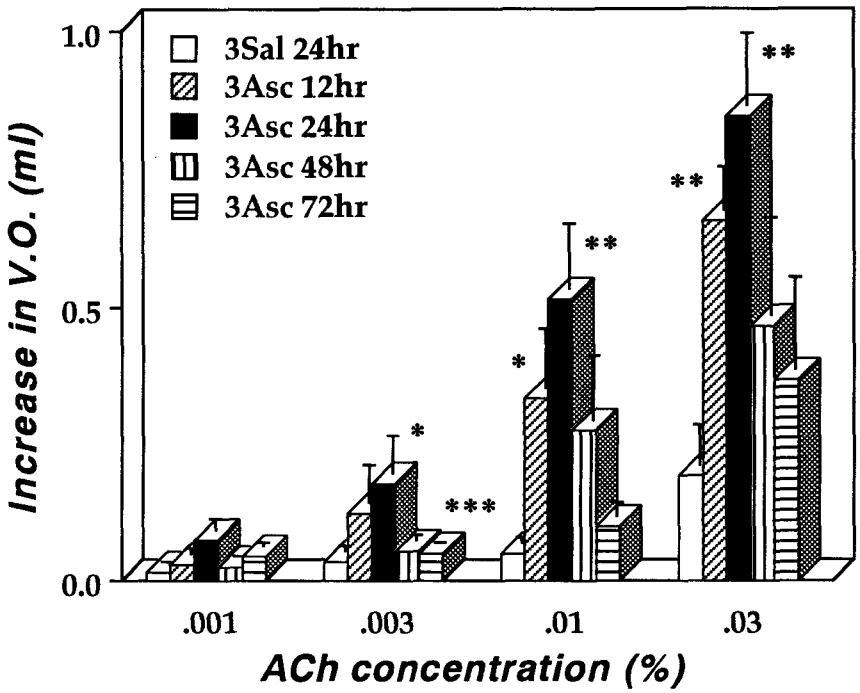


Figure 1-6

The time course changes in response to acetylcholine (ACh) after the last DNP-*Ascaris* antigen (Asc) challenge in anesthetized rats. Each point represents the mean with S.E. from 4 to 8 experiments. V.O.: ventilation overflow as an index of bronchoconstriction. *p<0.05, **p<0.01 and ***p<0.001 vs. control group.

Table 1-2. The passive cutaneous anaphylaxis (PCA) titers at various time points

	n	mean PCA titer	PCA range
Day 8	**5**	**21.2**	**2-64**
Day 12	**5**	**30.4**	**8-64**
Challenge after			
12 hr	**5**	**22.8**	**2-64**
24 hr	**14**	**40.0**	**8-256**
48 hr	**5**	**32.4**	**2-128**
72 hr	**7**	**12.3***	**2-32**

PCA titer was expressed as the mean of the end points determined as described in **Materials and methods**. Day 8: the day just before the first antigenic challenge. Day 12: the day just before the last antigenic challenge. Challenge after 12, 24, 48 and 72 hr: 12, 24, 48 and 72 hr after the last antigenic challenge. *$p<0.05$ vs. challenge after the 24-hr group (Wilcoxon rank-sum test).

Experiment 1-5: Effect of repeated antigenic challenge on *in vitro* airway responsiveness in actively sensitized rats.

Materials and methods

Antigen challenge

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were used. Animals were sensitized, boosted and repeatedly challenged with DNP-Asc as described in **Experiment 1-2**. Control rats received the same immunization, but inhaled saline instead of the DNP-Asc challenge dose.

In vitro determination of airway responsiveness

Twenty-four hours after the last antigenic challenge, the rats were sacrificed by a blow to the head and exsanguinated. The trachea and bronchus were rapidly removed, cleaned of adhering connective tissues and cut open longitudinally along its ventral surface. About a 5-mm length of the tracheal strip was isolated 5 mm under the larynx (5-6 cartilages), and the left main bronchial strip was also isolated (about 5-mm length, 8-9 cartilages). Each tissue strip was then suspended in a 10-ml organ bath, at a resting tension of 1.0 g. The isometrical contraction of each strip was measured with a force-displacement transducer (TB-612T, Nihon Kohden) and recorded on a polygraph (RM-85, Nihon Kohden). The organ bath contained modified Krebs-Henseleit solution with the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10.0 mM glucose. The buffer solution was maintained at 37 °C and oxygenated with 95 % O_2-5 % CO_2. During an equilibration period in the organ bath, the tissues were washed three to four times at 15 to 20 min intervals and equilibrated slowly to a baseline tension of 1.0 g for both strips. A further period of 15 min was required for tissue stabilization. The concentration-response curves for ACh and serotonin (5-HT) were constructed cumulatively, with a concentration range of 10^{-7}-10^{-4} M. A higher concentration of agonist was successively added after attainment of a plateau response to the previous concentration. Under these conditions, reproducible concentration-response curves were obtained on the same tissue strips.

In our *in vitro* experiments, NKA (10^{-8}-10^{-6} M) had no effect on most of the tracheal and main bronchial strips, so that we used 5-HT instead of NKA as a pharmacological stimulant in the *in vitro* experiments.

Drugs

The following drugs were used: acetylcholine chloride (purchased from Daiichi Pharmaceutical Co., Ltd., Tokyo) and serotonin (5-hydroxytriptamine hydrochloride).

Just before use in the *in vitro* studies, fresh solutions of ACh and serotonin were made in Krebs-Henseleit solution (composition described above).

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

Results

ACh elicited a concentration-dependent contractile response of the rat tracheal and bronchial strips (Figs. 1-7 and 1-8). In the control (saline inhalation) group, the contractile response elicited by 10^{-4} M ACh was 1.11±0.06 g (n=8) in the trachea (Fig. 1-7) and 0.38±0.05 g (n=8) in the bronchus (Fig. 1-8). As compared with the control group, the contractile response of the bronchial strips was significantly enhanced 24 hr after the last antigenic challenge in the repeated challenge group; the contractile response elicited by 10^{-4} M ACh was 1.00±0.08 g (n=6, $p<0.001$). No change in responsiveness of the tracheal strip (n=6) was, however, observed even after the antigenic challenge.

5-HT elicited relatively small contractile responses of the rat tracheal and bronchial strips (Figs. 1-9 and 1-10). In the control group, the maximal contractile response elicited by 10^{-4} M 5-HT was 0.07±0.03 g (n=5) in the tracheal strips (Fig. 1-9) and that elicited by $3x10^{-5}$ M 5-HT in the bronchial strips was 0.05±0.01 g (n=5) (Fig. 1-10). The contractile responses caused by 5-HT were also significantly enhanced in the bronchial strips which were excised 24 hr after the last antigenic challenge; the maximal contractile response elicited by $3x10^{-5}$ M 5-HT was 0.19±0.04 g (n=5, $p<0.05$).

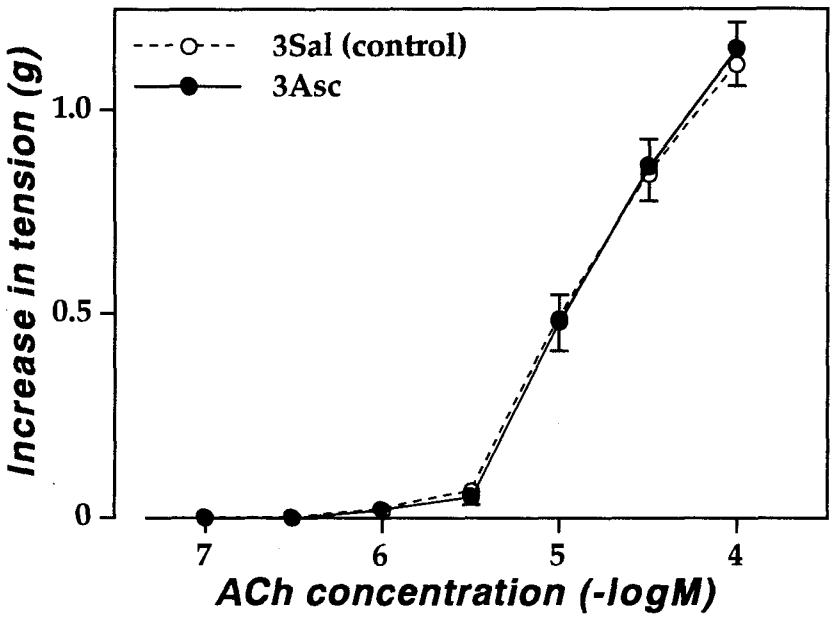


Figure 1-7

The concentration-response curves of contractile responses of the isolated trachea to acetylcholine (ACh) after 3 times repeated challenge with DNP-*Ascaris* antigen (Asc) or saline (Sal) inhalation in sensitized rats. Each point represents the mean with S.E. from 8 (control: 3Sal) and 6 (3Asc) experiments.

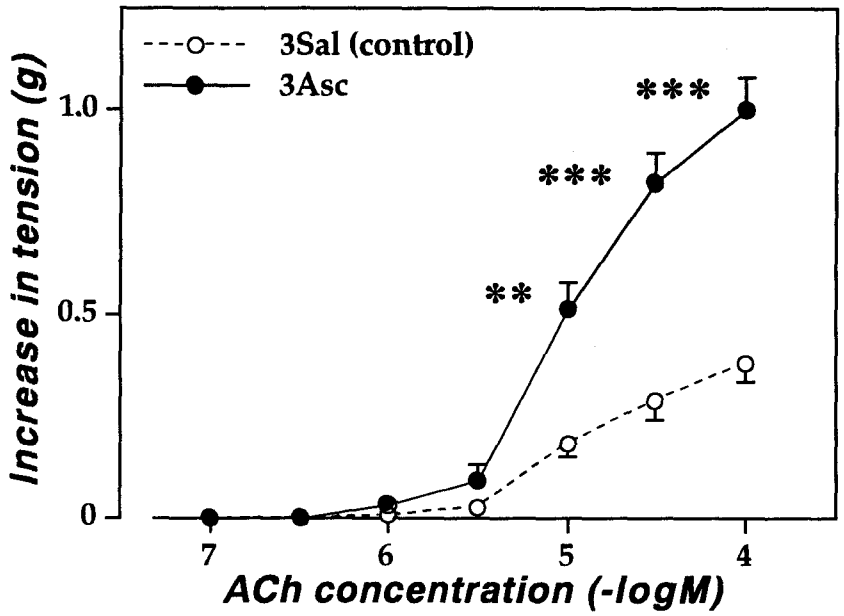


Figure 1-8

The concentration-response curves of contractile responses of the isolated bronchus to acetylcholine (ACh) after 3 times repeated challenge with DNP-*Ascaris* antigen (Asc) or saline (Sal) inhalation in sensitized rats. Each point represents the mean with S.E. from 8 (control: 3Sal) and 6 (3Asc) experiments. **p<0.01 and ***p<0.001 vs. control group.

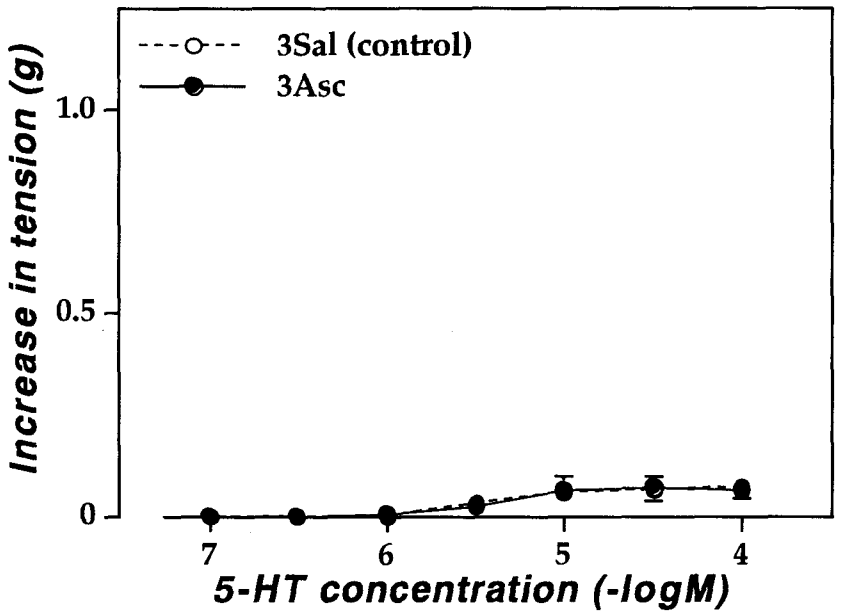


Figure 1-9

The concentration-response curves of contractile responses of the isolated trachea to serotonin (5-HT) after 3 times repeated challenge with DNP-*Ascaris* antigen (Asc) or saline (Sal) inhalation in sensitized rats. Each point represents the mean with S.E. from 5 (control: 3Sal) and 5 (3Asc) experiments.

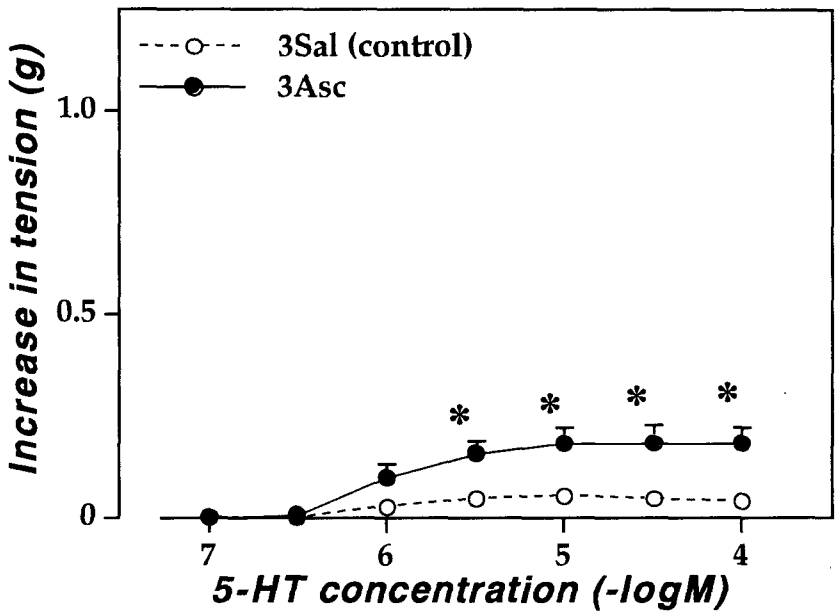


Figure 1-10

The concentration-response curves of contractile responses of the isolated bronchus to serotonin (5-HT) after 3 times repeated challenge with DNP-*Ascaris* antigen (Asc) or saline (Sal) inhalation in sensitized rats. Each point represents the mean with S.E. from 5 (control: 3Sal) and 5 (3Asc) experiments. *$p<0.05$ vs. control group.

Experiment 1-6: Effect of repeated antigenic challenge on airway microvascular leakage.

Materials and methods

Antigen challenge

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were used. Animals were sensitized, boosted and singly and/or repeatedly challenged with DNP-Asc as described in **Experiment 1-1** and **1-2**. Control rats received the same immunization, but inhaled saline instead of the DNP-Asc challenge dose.

Determination of airway microvascular leakage

At twenty-four hour or other time points after the last antigenic challenge, the rats were anesthetized with urethane (2 g/kg, i.p.). Airway vascular permeability was quantified by the extravasation of Evans blue dye (E.B.) using the modification of the method of Evans *et al.* (1987), which correlates well with extravasation of radiolabeled albumin (Udaka *et al.*, 1970). E.B. (30 mg/ml in saline, filtered with paper filter), 30 mg/kg, and heparin (600 U/kg) were administered by *i.v.* -injection. Twenty minutes later, the animals were exsanguinated from the abdominal aorta, and then the thorax was opened and a blunt-ended 13-gauge needle was passed through the right ventricle into the pulmonary artery. The pulmonary veins were incised to allow the outflow of perfusate, and the airways and lungs were perfused with 20-30 ml of 1 % paraformaldehyde in citrate-buffered saline (pH 3.5) to remove intravascular dye and to fix the tissues. After then, the trachea, main bronchus and lungs were removed and their wet weights were measured.

E.B. was extracted by incubating tissues in 1 ml (trachea and main bronchus) or 3 ml (lungs) of 100 % formamide at 60 °C for 24 hr, and its concentration was determined by light absorbance at 620 nm (U-2000 spectrophotometer, Hitachi) and interpolation on a standard curve of E.B. concentrations (3.125-50 μg/ml). E.B. content from each sample was expressed as nanograms per milligram wet weight of tissue.

Drugs

Evans blue was obtained from Merck Co., Ltd. (New Jersey, USA) and was dissolved in saline and stocked at room temperature. Heparin sodium was from Kodama Co., Ltd. (Tokyo) and was diluted freshly in saline each day.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

Results

Figure 1-11 shows E.B. extravasation in the tracheal, main bronchial and lung tissues at 24 hr after the single challenge and at 12 or 24 hr after the last challenge of the 3 times repeated challenge. E.B. extravasation in the trachea was significantly increased at 24 hr after the repeated challenge ($p<0.05$ vs. control). In the main bronchus, E.B. extravasation was significantly increased at 24 hr after the single challenge ($p<0.01$ vs. control), but a more marked increase in E.B. extravasation was observed at 12 and 24 hr after the repeated challenge ($p<0.001$ and $p<0.01$ vs. single challenge group, respectively). On the other hand, E.B. extravasation in the lungs was increased in none of the challenge groups.

The E.B. extravasations in the tracheal (12.16±4.15 ng/mg tissue, n=5), main bronchial (11.65±2.00 ng/mg tissue, n=5) and lung (3.62±0.24 ng/mg tissue, n=5) tissues 48 hr after the last antigenic challenge were not significantly different from the respective control value (data not shown).

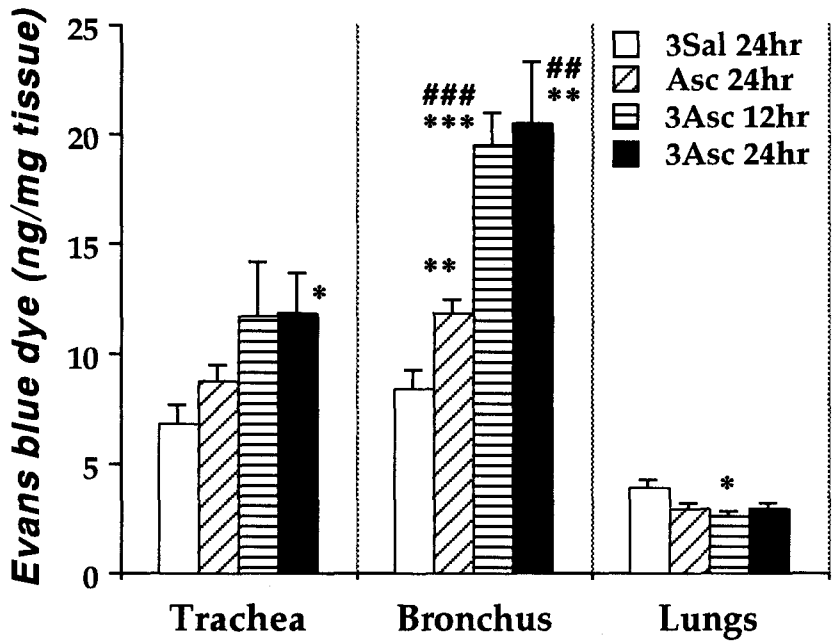


Figure 1-11

The changes in amount of extravasated Evans blue dye (ng Evans blue dye /mg wet weight tissue) in the rat trachea (left panel), main bronchus (middle panel) and lungs (right panel). Each column represents the mean with S.E. from 8 (control: 24 hr after inhaling saline), 11 (24 hr after the single antigenic challenge), 5 (12 hr after the last antigenic challenge) and 8 (24 hr after the last antigenic challenge) experiments. *$p<0.05$, **$p<0.01$ and *** $p<0.001$ vs. control group. ##$p<0.01$ and ###$p<0.001$ vs. single challenge group.

Experiment 1-7: Histological changes in airway tissues after repeated antigenic challenge.

Materials and methods

Antigen challenge

Specific-pathogen-free male Wistar rats weighing 230-314 g (6-8 weeks of age), purchased from Charles River Japan, Inc., were used. Animals were sensitized, boosted and singly and/or repeatedly challenged with DNP-Asc as described in **Experiment 1-1** and **1-2**. Control rats received the same immunization, but inhaled saline instead of the DNP-Asc challenge dose.

Histological examination

For histological examination, 5 to 7 rats of each group (control, single challenge and repeated challenge groups) were used. These animals were exsanguinated from the abdominal aorta, and the lower airway was removed after opening thorax. Then the tissue was gently washed with normal saline and fixed in neutral formalin solution. The trachea, main bronchus and lung were cut into 3-5 mm chips, and each of the chips was embedded in paraffin by a standard technique. The paraffin sections were stained with hematoxylin and eosin.

Results

Figures 1-12 and 1-13 show the main bronchial tissues stained with hematoxylin and eosin from the control and repeatedly challenged rats, respectively. Twenty-four hours after the repeated challenge, mast cell and inflammatory cell (monocytes, lymphocytes, polynuclear cells, etc.) infiltration was markedly increased. The infiltration of these cells was more severe in the repeated challenge group (Fig. 1-13) than in the single challenge group (data not shown). In the repeated challenge group, the main bronchus was affected more severely than the trachea and lungs. Furthermore, epithelial cells were moderately changed (*e.g.*, increased and hypertrophied goblet cells), especially in the main bronchus.

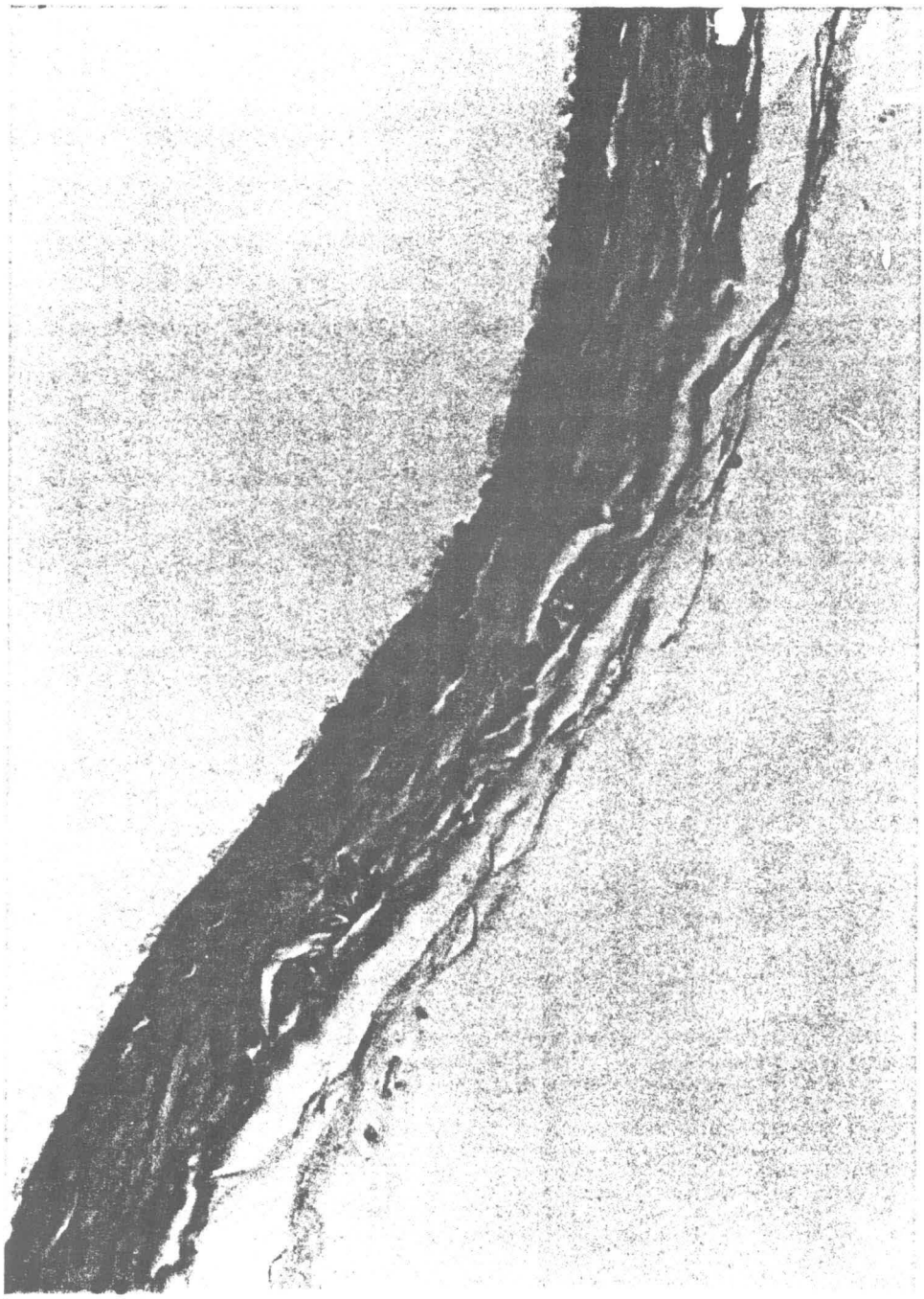


Figure 1-12

A photograph showing the main bronchus from the control rat (without antigenic challenge) (×400). Hematoxylin-eosin staining.

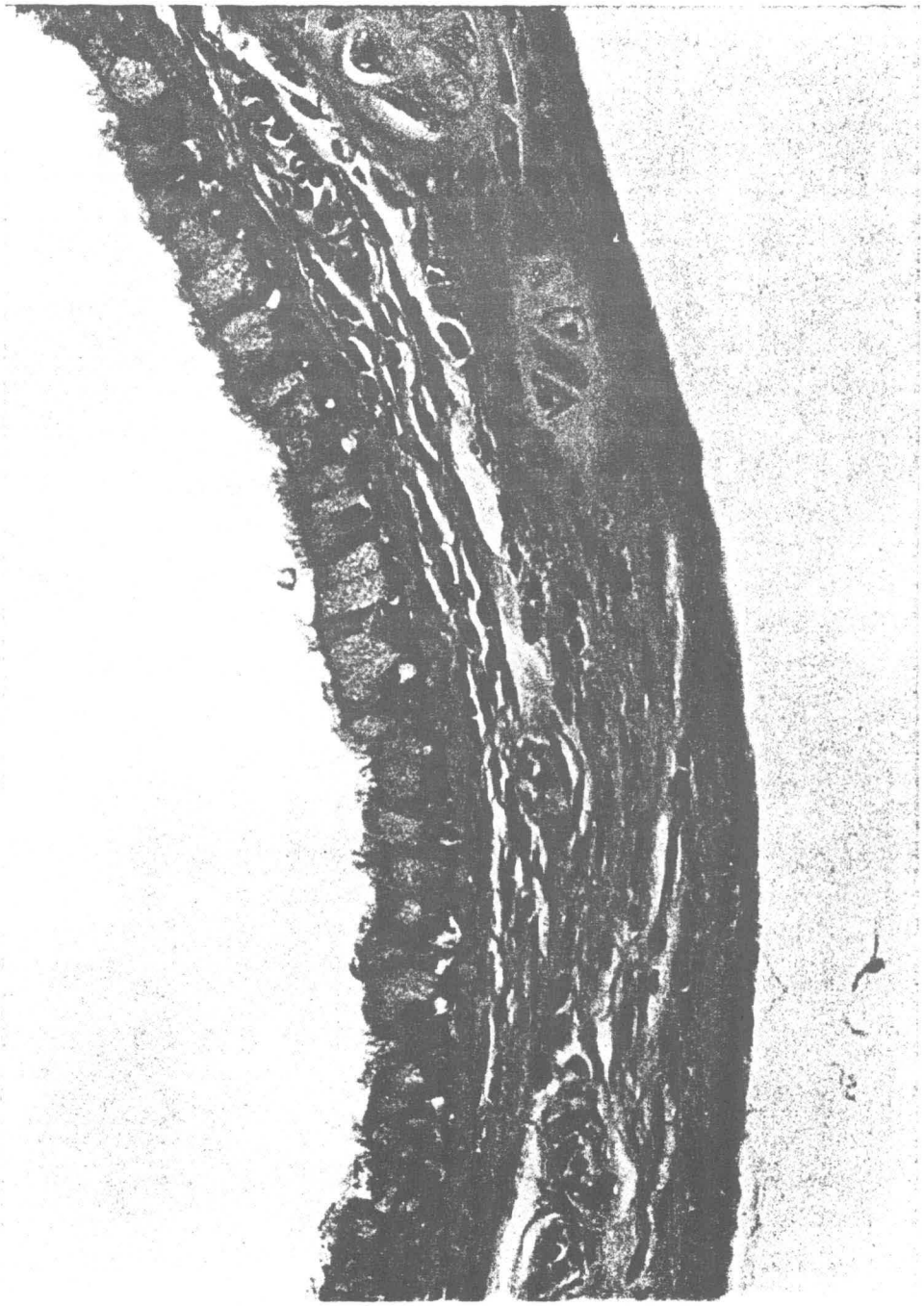


Figure 1-13

A photograph showing the main bronchus from the repeatedly antigen challenged rat (×400). Hematoxylin-eosin staining.

Discussion

Our results demonstrated that repeated antigenic challenge caused a distinct AHR (enhanced airway responsiveness to inhaled ACh and NKA) and airway inflammation (increased airway microvascular leakage and histological change) in actively sensitized rats. The peak of AHR to ACh was shown at 24 hr after the last antigenic challenge. At this time point, it was found that the AHR could be obtained even in the *in vitro* level, that is, in bronchial tissue. A severe airway inflammation was demonstrated at 12-24 hr after the last antigenic challenge in the bronchus.

ACh and NKA were used to determine the *in vivo* airway responsiveness. ACh was frequently used for the test of AHR in clinical diagnosis. NKA, one of the putative neurotransmitters of the excitatory non-adrenergic, non-cholinergic nervous system, is a potent bronchoconstrictor agent (Solway & Leff, 1991). In the present study, the AHR to ACh was completely blocked by atropine (data not shown), indicating that there existed a muscarinic hyperreactivity. However, this is not a specific hyperreactive response because the airway responsiveness to NKA was also enhanced.

When rats were sensitized with DNP-Asc antigen according to the method of Tada and Okumura (1971), the 48 hr PCA titer (IgE antibodies are involved) is known to reach the maximal level at 8 days after the first immunization. Thus, at first, this time point was selected to carry out the antigenic challenge (to elicite the immediate asthmatic response) under unanesthesia. The objective symptoms observed were analogous to those of allergic bronchial asthma (*e.g.*, abnormality of breaths) in all the animals used.

Presently, the airway responsiveness to ACh and NKA were significantly enhanced at 24 hr after the single challenge, but the enhanced level was not so potent. Generally, with regard to allergic bronchial asthmatics, they are continually exposed to a certain antigen (*e.g.*, house dust). Recently, with this view in mind, repeated antigenic challenge to sensitized animals has been attempted by some investigators (Bellofiore & Martin, 1988; Gundel *et al.*, 1990; Kleeberger *et al.*, 1985). On the other hand, Bellofiore and Martin (1988) reported that the gradual desensitization to OA antigen was elicited after 6 times repeated challenge in Brown-Norway rats. In our experiments, the antigenic challenge protocol of 3 times repeated challenge every 48 hr successfully produced a marked increased responsiveness to ACh and NKA 24 hr after the last challenge of the repeated challenge.

Twenty-four hours after the last antigenic challenge, the in vitro airway responsiveness to ACh and 5-HT were also enhanced in the main bronchus but not in the trachea. The airway microvascular leakage was more markedly increased in the bronchus than in the trachea. Furthermore, in our histological study, infiltration of inflammatory cells was much more

severe in the bronchus than in the trachea. It has been thought that airway inflammation is induced by some chemical mediators such as prostaglandins, leukotrienes (LTs), thromboxane A_2 (TXA_2), and platelet-activating factor (PAF) derived from the inflammatory cells (*e.g.*, mast cells, neutrophils, eosinophils), which results in AHR (O'Byrne, 1986; Chung, 1986). Additionaly, neuropeptides which are contained in the sensory nerve endings (Barnes, 1986), may be also involved in the occurrence of airway inflammation. The difference in the present results between the tracheal and main bronchial regions is not now explainable. It was, however, possible that because the distribution of these inflammatory cells might be more abundant in the main bronchus, the chemotaxis of these inflammatory cells might be caused more strongly in the main bronchus than in the trachea. Another possibility is that the neural control of the main bronchus might be essentially different from that of the trachea (*e.g.*, the regional difference of responsiveness to ACh, carbachol (Vornanen & Tirri, 1981) and LTC_4 (Szarek & Evans, 1988) was reported in rats and excitatory nonadrenergic, noncholinergic innervation is much abundant in the bronchial region than in the tracheal region in guinea pigs (Stretton *et al.*, 1992)). Furthermore, it is possible that there may be a difference in physical adhesion of the antigen to the airway lumenal surface as compared to its adhesion in the bronchus. This regional difference inthe airway inflammation was likely to reflect the regional difference in the AHR in the *in vitro* experiments also.

It has been reported that an antigenic challenge to sensitized guinea pigs (Ishida *et al.*, 1990) and rabbits (Tanaka *et al.*, 1991) resulted in an airway smooth muscle hyperresponsiveness *in vitro*. These animals also obtained AHR in the *in vivo* level (Ishida *et al.*, 1989; Tanaka *et al.*, 1991). These findings therefore suggest that the increased airway smooth muscle responsiveness itself may be reflected in the *in vivo* AHR. Our results that the responsiveness to ACh and 5-HT were enhanced in the bronchial tissue *in vitro* supports this speculation. However, attempts to demonstrate hyperresponsiveness of isolated airway smooth muscle strips from asthmatics *in vitro* have so far produced varied results. There seems to be little or no increase in the sensitivity of airway strips from asthmatics (obtained post-mortem) (Goldie *et al.*, 1986; Cerrina *et al.*, 1986). In contrast, some studies, particulaly those using surgical specimens, have described a substantial increase in muscle responsiveness (Schellenberg & Foster, 1984; de Jongste *et al.*, 1987; Bai, 1990).

In our findings, a close relationship between the bronchial inflammation and the AHR was demonstrated. That is, the peak of the airway inflammation was shown at 12-24 hr after the last antigenic challenge and the peak of the AHR was shown at 24 hr after the last challenge. When dogs inhaled ozone, a close association was observed between the development of AHR and an acute inflammatory response in the airways, as measured by an

influx of neutrophils into the epithelial and sub-epithelial layers of the airways (Holtzman *et al.*, 1983) and bronchoalveolar fluid (Fabbri *et al.*, 1984). Furthermore, in other animal preparations such as rabbits (Marsh *et al.*, 1985), guinea pigs (Murlas & Roum, 1985) and even in humans (Seltzer *et al.*, 1986), airway inflammation had also occurred at the same time as the development of AHR. Gibson *et al.* (1991) reported that stable asthmatic individuals who were subjected to the provocation test with allergen inhalation demonstrated an increased histamine airway responsiveness at 24 hr after the provocation, accompanied by airway inflammation. Their results are in good accordance with ours in terms of time course. In the present study, the airway inflammation (as evaluated by the E.B. exudation and histological examination) was induced in the main bronchus after the single antigenic challenge to sensitized rats, and a more severe inflammatory reaction was induced after the repeated antigenic challenge. It is likely that the antigenic challenge to sensitized rats caused an airway inflammation which was followed by AHR.

The present rat AHR model is considered to be useful for studying the underlying mechanisms of pathogenesis of AHR and for screening therapeutic drugs for AHR.

CHAPTER 2:
Strain differences in airway hyperresponsiveness after repeated antigenic challenge

Introduction

As described in **CHAPTER 1**, we developed an animal model of AHR by using male Wistar rats that were actively sensitized with DNP-*Ascaris* antigen (DNP-Asc) and challenged 3 times by inhaling the aerosolized antigen. It is documented that there is a strain difference in immediate asthmatic response caused by DNP-*Ascaris* antigen in rats (Misawa *et al.*, 1987). To study the mechanisms involved in nonspecific airway hyperresponsiveness (AHR), the selection of a suitable strain is important. There has however been no report in which AHR response was compared among strains of the rat. In the present study, we therefore investigated the strain differences in liability for AHR among the three strains of rats: Brown-Norway (BN), a high IgE responder strain (Pauwels *et al.*, 1978; Pauwels *et al.*, 1979), Long-Evans Cinnamon (LEC), a new mutant which develops spontaneous hepatitis (Yoshida *et al.*, 1987) and has dysfunction of helper T cells (Agui *et al.*, 1990; Yamada *et al.*, 1991) and Wistar.

Experiment 2-1: Strain differences in *in vivo* airway responsiveness to acetylcholine after repeated antigenic challenge.

Materials and methods

Animals

Male Wistar rats (6 weeks of age, 170-190 g) were purchased from Charles River Japan, Inc. and male Brown-Norway rats (BN; 6 weeks of age, 120-150 g) and male Long-Evans Cinnamon rats (LEC; 6 weeks of age, 110-140 g) were kindly gifted from this company. All these rats were specific-pathogen-free and housed under standard laboratory conditions with free access to food and water.

Sensitization and antigenic challenge

The method for inducing AHR was described in **Experiment 1-2**. Animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract (DNP-Asc, 2 mg protein, *s.c.*) together with *Bordetella pertussis* ($2X10^{10}$) as an adjuvant and were boosted by DNP-Asc (0.5 mg protein, i.m.) 5 days later, according to the method of Tada and Okumura (1971). Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc (6 mg protein/ml, 5-6 ml) with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo) for 20 min under conscious state in a plexiglass box (300x200 mm, height: 150 mm). Then the animals received 3 times repeated antigenic challenge every 48 hr with the same challenge method described above. Control rats received the same immunization, but were inhaled saline instead of the DNP-Asc challenge.

In vivo determination of airway responsiveness

The determination of airway responsiveness to ACh was done 24 hr after the last antigenic challenge. Rats were anesthetized with urethane (2 g/kg, i.p.), placed in the supine position and ventilated artificially through a tracheal cannula at a frequency of 70 breaths/min. Respiratory volume was adjusted at the beginning of the experiment so that ventilation overflow (VO) was 0.5 ml in each rat. Spontaneous respiration was stopped by pancuronium bromide (1 mg/kg, i.v.). The bronchomotor tone was measured by the modified Konzett-Rös sler method (Konzett and Rössler, 1940) as described in **Experiment 1-1**. The lung was inflated at a fixed volume of air under a constant pressure (5 cmH_2O), and VO was continuously recorded with a combination of pneumotachograph (TP-602T, Nihon Kohden) and integrator (EI-601G, Nihon Kohden). Systemic blood pressure was monitored with a

pressure transducer (DX-300, Nihon Kohden) from a cannula inserted into the left carotid artery. All the above parameters were recorded using a polygraph (RM-6000, Nihon Kohden).

ACh (0.001-0.03 %) was cumulatively inhaled each for 3 min to the rats by aerosolizing the solution contained in a specially devised plastic cylindrical chamber (capacity: 25 ml) which was introduced in an ultrasonic nebulizer (TUR-3200, Nihon Kohden). The ultrasonic nebulizer with the plastic chamber was placed into the respiratory circuit for the aerosolized mist being inhaled into the airway at each time of ventilation.

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo) and urethane (Sigma, St. Louis, USA).

ACh and pancuronium bromide were dissolved and/or diluted in saline at the usage.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA).

Results

At each antigenic challenge, the symptoms (e.g. abnormality of breaths) were observed in the animals. The symptoms observed were remarkable in degree in Wistar and Brown-Norway (BN) rats, but were moderate in Long-Evans Cinnamon (LEC) rats.

In all the three strains of rats, inhalation of four cumulative aerosols of ACh (0.001, 0.003, 0.01 and 0.03 %; each for 3 min) to anesthetized normal (non-sensitized) rats induced a concentration-related increase in ventilation overflow (V.O.), that is, a bronchoconstriction (Figs. 2-1, 2-2 and 2-3). The normal rats of BN strain were most sensitive to ACh among respective normal rats of the three strains ($F_{[1,40]}=6.291$, $p<0.05$ vs. Wistar).

In Wistar rats, the sensitization itself was without effect on the airway responsiveness to ACh. However, the concentration-response curve to inhaled ACh was markedly shifted to the left 24 hr after the last antigenic challenge ($F_{[1,45]}=18.492$, $p<0.01$). The airway responsiveness to ACh was significantly enhanced at concentrations of 0.001 % ($p<0.05$ vs. normal and sensitized control) and 0.03 % ($p<0.05$ vs. normal and $p<0.01$ vs. sensitized control) (Fig. 2-1). In BN rats, the concentration-response curve to ACh of the sensitized control group was slightly shifted to the left, although not significant, as compared with the

normal group. Twenty-four hr after the last antigenic challenge, no enhancement was observed when compared with the sensitized control group. The airway responsiveness to ACh was, however, significantly shifted to the left ($F_{[1,55]}$=13.042, $p<0.01$), and enhanced at a concentration of 0.03 % ($p<0.05$) as compared with the normal group (Fig. 2-2). On the other hand, no significant change in responsiveness to ACh was observed in LEC rats after the sensitization or repeated antigenic challenge (Fig. 2-3).

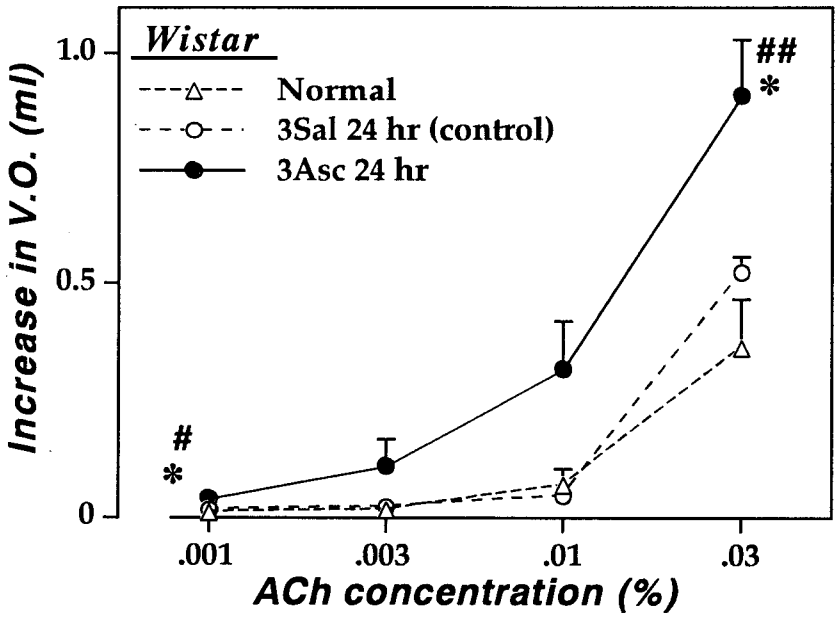


Figure 2-1

The concentration-response curves of bronchomotor response to aerosolized acetylcholine (ACh) after 3 times repeated challenge with DNP-Ascaris antigen (Asc) in sensitized Wistar rats. The airway responsiveness to cumulatively inhaled ACh was determined 24 hr after the last antigenic challenge. Δ; nonsensitized control group (Normal). O; animals that were sensitized and inhaled saline (Sal) 3 times instead of Asc (control). ●; animals that were sensitized and challenged 3 times (3Asc 24 hr). Each point represents the mean with S.E. from 4-8 experiments. *$p<0.05$ vs. Normal group, and #$p<0.05$ and ##$p<0.01$ vs. control group.

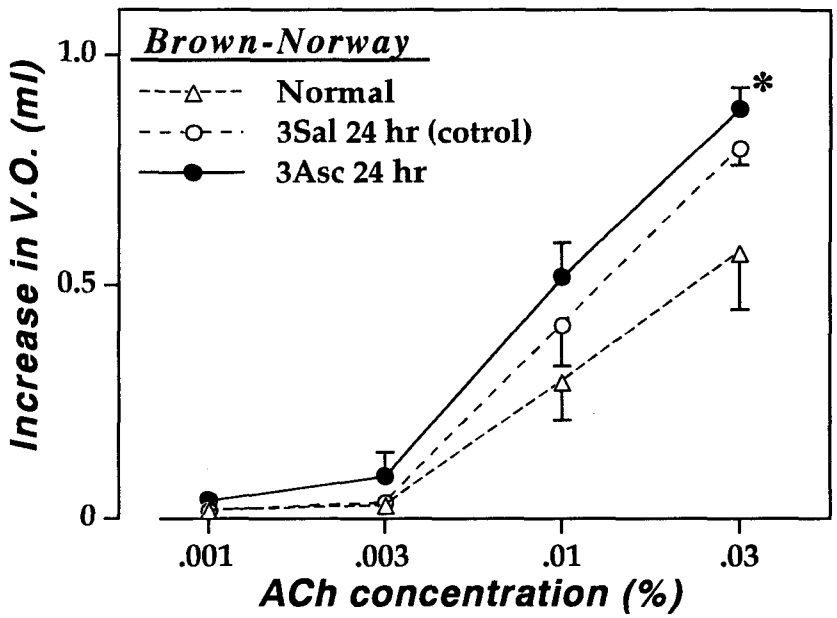


Figure 2-2

The concentration-response curves of bronchomotor response to aerosolized acetylcholine (ACh) after 3 times repeated challenge with DNP-Ascaris antigen (Asc) in sensitized Brown-Norway rats. The airway responsiveness to cumulatively inhaled ACh was determined 24 hr after the last antigenic challenge. △; nonsensitized control group (Normal). ○; animals that were sensitized and inhaled saline (Sal) 3 times instead of Asc (control). ●; animals that were sensitized and challenged 3 times (3Asc 24 hr). Each point represents the mean with S.E. from 4-8 experiments. *$p<0.05$ vs. Normal group.

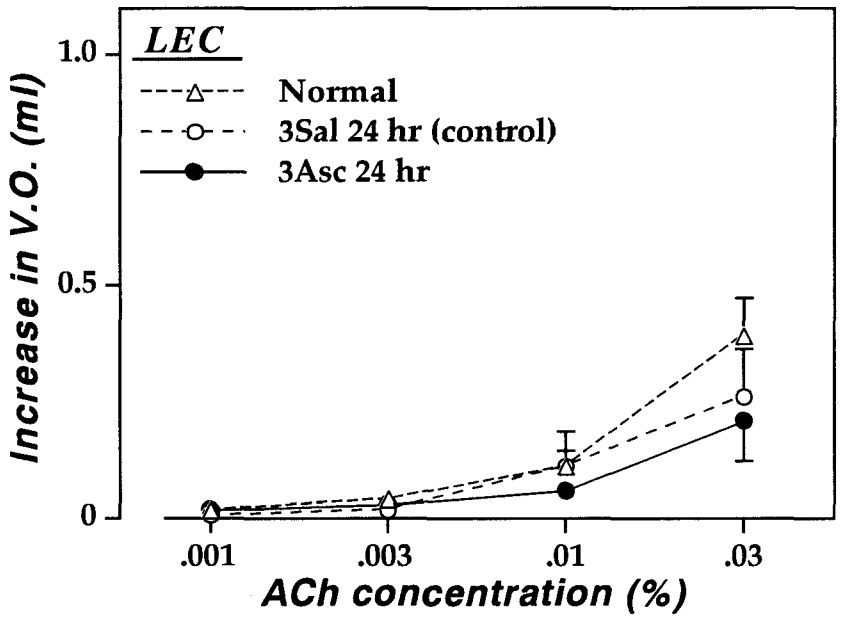


Figure 2-3

The concentration-response curves of bronchomotor response to aerosolized acetylcholine (ACh) after 3 times repeated challenge with DNP-Ascaris antigen (Asc) in sensitized Long-Evans Cinnamon (LEC) rats. The airway responsiveness to cumulatively inhaled ACh was determined 24 hr after the last antigenic challenge. △; nonsensitized control group (Normal). O; animals that were sensitized and inhaled saline (Sal) 3 times instead of Asc (control). ●; animals that were sensitized and challenged 3 times (3Asc 24 hr). Each point represents the mean with S.E. from 4-8 experiments. *$p<0.05$ vs. Normal group.

Experiment 2-2: Strain differences in *in vitro* airway responsiveness to acetylcholine and electrical field stimulation after repeated antigenic challenge.

Materials and methods

Sensitization and antigenic challenge

Male Wistar, Brown-Norway (BN) and Long-Evans Cinnamon (LEC) rats were sensitized and received repeated antigenic challenge by the method described in **Experiment 2-1**.

In vitro determination of airway responsiveness

Immediately after the *in vivo* study as described in **Experiment 2-1**, animals were thoracotomized and bled from the heart to collect the antisera under anesthesia, and the trachea and bronchus were rapidly removed and cleaned of adhering connective tissues. About 5 mm length of the left main bronchus was isolated (8-9 cartilages) and the resultant tissue ring preparation was then suspended in a 10 ml organ bath at a resting tension of 0.5 g. The isometrical contraction was measured with a force-displacement transducer (TB-612T, Nihon Kohden) and recorded on a polygraph (RM-85, Nihon Kohden). The organ bath contained modified Krebs-Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. The buffer solution was maintained at 37 °C and oxygenated with 95 $\%O_2$-5 $\%CO_2$. During an equilibration period in the organ bath, the tissues were washed three to four times at 15 to 20 min intervals and equilibrated slowly to a base line tension of 0.5 g. Fifteen min after the last washing, the concentration-response curve to ACh (10^{-7}-10^{-3} M) was constructed cumulatively. A higher concentration of ACh was successively added after attainment of a plateau response to the previous concentration. After determination of ACh responsiveness, the tissues were frequently washed out at 15 to 20 min intervals. Then the airway responsiveness to electrical field stimulation (EFS; 20 V, 0.5 msec, 0.5-64 Hz for 15 sec) was also determined by using an electronic stimulator (SEN-3301, Nihon Kohden) after the equilibration period. A higher frequency of EFS was performed at 4 min intervals. Under these conditions, reproducible concentration- and frequency-response curves were obtained on the same tissue strip. In these *in vitro* studies, the data were expressed as increased tension per dry tissue weight (g/mg dry weight).

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo) and acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo).

ACh was dissolved and diluted in modified Krebs-Henseleit solution.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA).

Results

ACh elicited a concentration-dependent contractile response of the bronchial rings of all the three strains of rats (Figs. 2-4, 2-5 and 2-6). In Wistar rats, the sensitization was without effect on the airway responsiveness to ACh. However, the response to cumulatively administered ACh (10^{-7}-10^{-3} M) was significantly augmented 24 hr after the last antigenic challenge as compared with normal group ($F_{[1,81]}=6.794$, $p<0.05$) and sensitized control group ($F_{[1,81]}=11.242$, $p<0.01$) (Fig. 2-4). The contractile responses elicited by 10^{-3} M ACh were the following: normal group; 1.49±0.29 g/mg dry weight (n=5), sensitized control group; 1.26±0.31 g/mg dry weight (n=5), and repeatedly challenged group; 2.16±0.37 g/mg dry weight (n=6). On the other hand, neither the sensitization nor the repeated antigenic challenge had significant effect on the airway responsiveness to ACh in BN and LEC rats (Figs. 2-5 and 2-6).

Figures 2-7, 2-8 and 2-9 illustrates the frequency-response curves for EFS response of the isolated left main bronchi of normal, sensitized and repeated challenged rats. EFS elicited a frequency-dependent contractile response of the bronchial tissues of the strains; the contractile responses elicited by EFS were completely abolished by tetrodotoxin (10^{-6} M) or atropine (10^{-6} M) (data not shown), indicating that these responses were mediated by neural, especially cholinergic component. In all the strains of rats, the sensitization was without significant effect on the EFS-induced contractile responses. However, the frequency-response curves were significantly shifted to the left after repeated antigenic challenge in Wistar ($F_{[1,72]}=20.936$, $p<0.01$) and BN rats ($F_{[1,80]}=32.351$, $p<0.01$), but not in LEC rats.

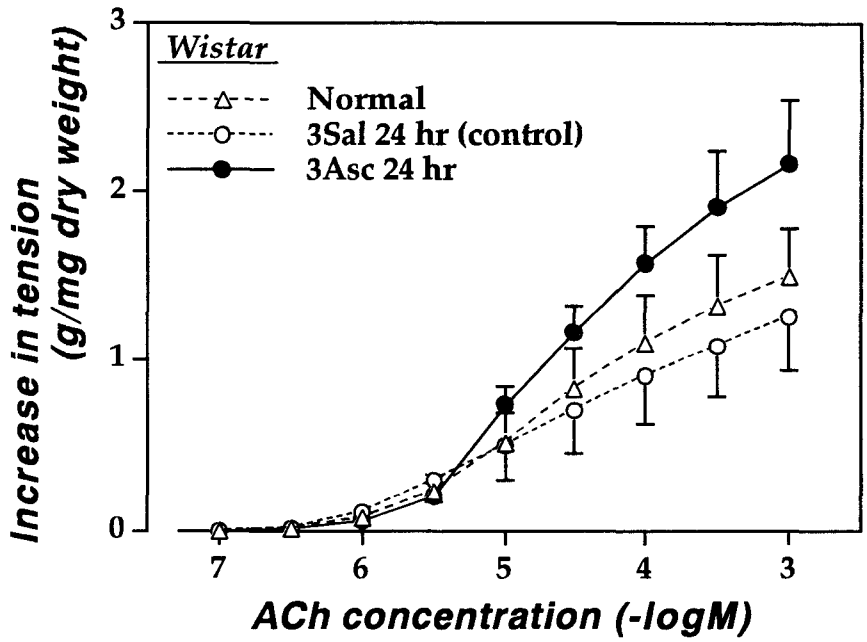


Figure 2-4

The concentration-response curves for contractile responses to acetylcholine (ACh) of the main bronchus isolated from 3 times repeated challenged Wistar rats with DNP-*Ascaris* antigen (3Asc 24 hr; ●) or from 3 times saline inhaled Wistar rats that had been sensitized (3Sal 24 hr; ○). *Open triangle* (△); non-sensitized control group (Normal). Each point represents the mean with S.E. from 4-8 experiments. Twenty-four hours after the last challenge, the concentration-response curve was significantly augmented ($p<0.05$ vs. Normal and $p<0.01$ vs. control by ANOVA).

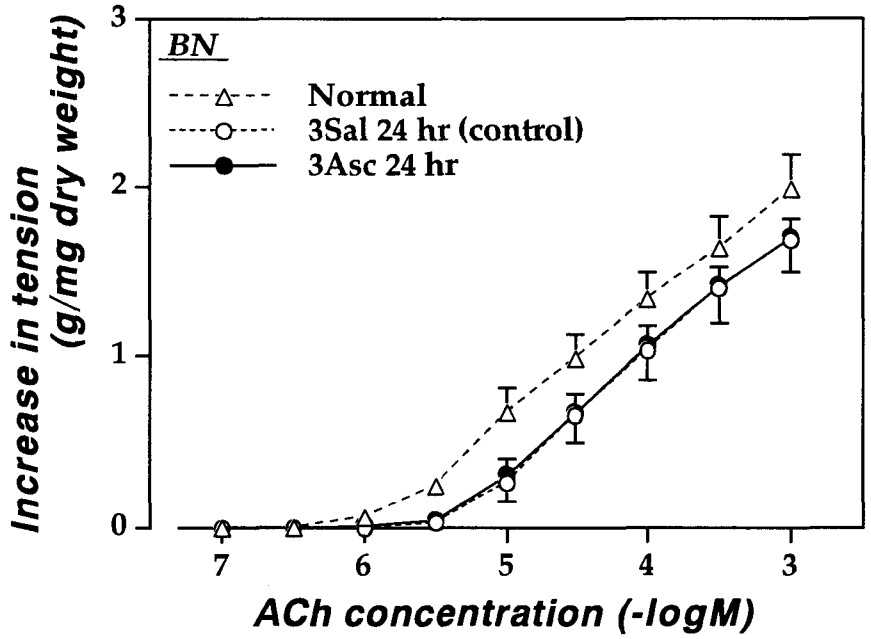


Figure 2-5

The concentration-response curves for contractile responses to acetylcholine (ACh) of the main bronchus isolated from 3 times repeated challenged Brown-Norway (BN) rats with DNP-*Ascaris* antigen (3Asc 24 hr; ●) or from 3 times saline inhaled BN rats that had been sensitized (3Sal 24 hr; ○). *Open triangle* (△); non-sensitized control group (Normal). Each point represents the mean with S.E. from 4-8 experiments. No significant difference among the groups was observed in BN rats.

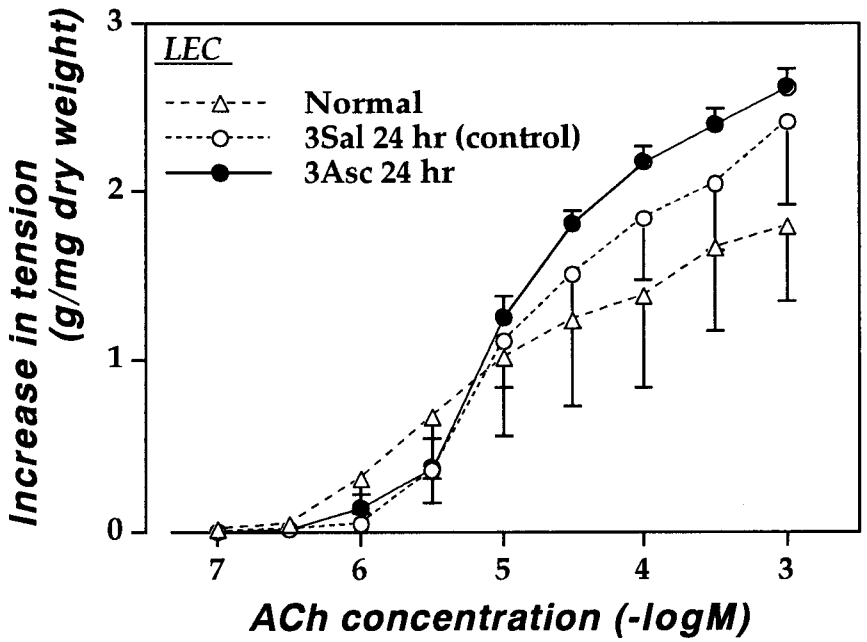


Figure 2-6

The concentration-response curves for contractile responses to acetylcholine (ACh) of the main bronchus isolated from 3 times repeated challenged Long-Evans Cinnamon (LEC) rats with DNP-*Ascaris* antigen (3Asc 24 hr; ●) or from 3 times saline inhaled LEC rats that had been sensitized (3Sal 24 hr; ○). *Open triangle* (△); non-sensitized control group (Normal). Each point represents the mean with S.E. from 4-8 experiments. No significant difference among the groups was observed in LEC rats.

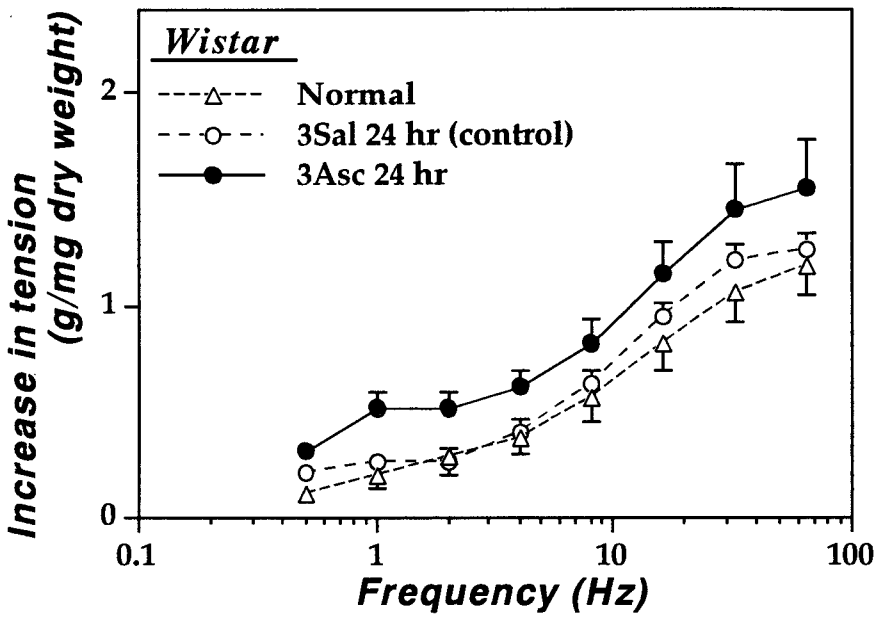


Figure 2-7

The frequency-response curves for contractile responses to electrical field stimulation (20 V, 0.5 msec, 0.5-64 Hz for 15 sec) of the main bronchus isolated from 3 times repeated challenged Wistar rats with DNP-*Ascaris* antigen (3Asc 24 hr; ●) or from 3 times saline inhaled Wistar rats that had been sensitized (3Sal 24 hr; ○). Open triangle (△); non-sensitized control group (Normal). Each point represents the mean with S.E. from 4-8 experiments. Twenty-four hours after the last challenge, the frequency-response curves were significantly shifted to the left ($p<0.01$ vs. Normal or control by ANOVA).

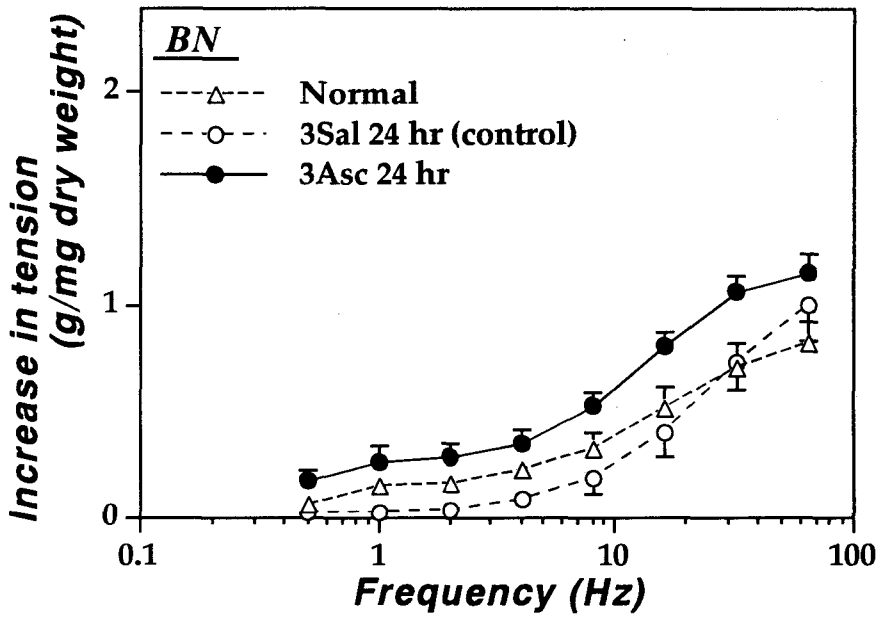


Figure 2-8

The frequency-response curves for contractile responses to electrical field stimulation (20 V, 0.5 msec, 0.5-64 Hz for 15 sec) of the main bronchus isolated from 3 times repeated challenged Brown-Norway (BN) rats with DNP-*Ascaris* antigen (3Asc 24 hr; ●) or from 3 times saline inhaled BN rats that had been sensitized (3Sal 24 hr; ○). Open triangle (△); non-sensitized control group (Normal). Each point represents the mean with S.E. from 4-8 experiments. Twenty-four hours after the last challenge, the frequency-response curves were significantly shifted to the left ($p<0.01$ vs. Normal or control by ANOVA).

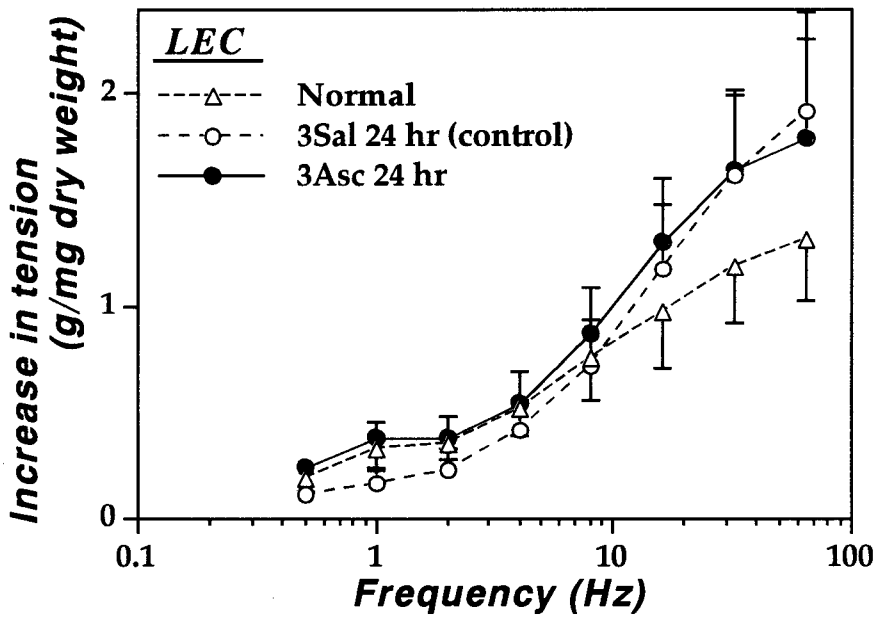


Figure 2-9

The frequency-response curves for contractile responses to electrical field stimulation (20 V, 0.5 msec, 0.5-64 Hz for 15 sec) of the main bronchus isolated from 3 times repeated challenged Long-Evans Cinnamon (LEC) rats with DNP-*Ascaris* antigen (3Asc 24 hr; ●) or from 3 times saline inhaled LEC rats that had been sensitized (3Sal 24 hr; ○). Open triangle (△); non-sensitized control group (Normal). Each point represents the mean with S.E. from 4-8 experiments. No significant difference among the groups was observed in LEC rats.

Experiment 2-3: Effect of repeated antigenic challenge on bronchial tissue wet to dry (wet/dry) weight ratio.

Materials and methods

Sensitization and antigenic challenge

Male Wistar, Brown-Norway (BN) and Long-Evans Cinnamon (LEC) rats were sensitized and received repeated antigenic challenge by the method described in **Experiment 2-1**.

Determination of bronchial tissue wet to dry (wet/dry) weight ratio

To determine the extent of the airway edema, the wet/dry weight ratio of each bronchial tissue was determined. After the *in vitro* study, the wet weight of the bronchial tissue was measured and then the tissue was placed in a freeze-dry system for 24 hr. Then the tissue dry weight was measured and the wet/dry weight ratio was calculated.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

Results

In Wistar rats, the dry tissue weights (mg) were the following: normal group; 1.02±0.06 (n=5), sensitized control group; 0.98±0.10 (n=5), and repeated challenged group; 0.92±0.08 (n=6); there was no significant difference among the groups. However, the wet/dry weight ratio of the left bronchial tissue from repeated challenged group (4.48± 0.34) was significantly increased as compared with that from sensitized control group (2.77±0.10, $p<0.01$) (Fig. 2-10). On the other hand, the wet/dry weight ratio of the left bronchial tissues from repeated challenge group of BN (4.05±0.35, n=7) or LEC rats (3.72± 0.14, n=7) had no significant changes as compared with respective sensitized control groups (3.69± 0.16 (n=7) and 3.04±0. 37 (n=5)).

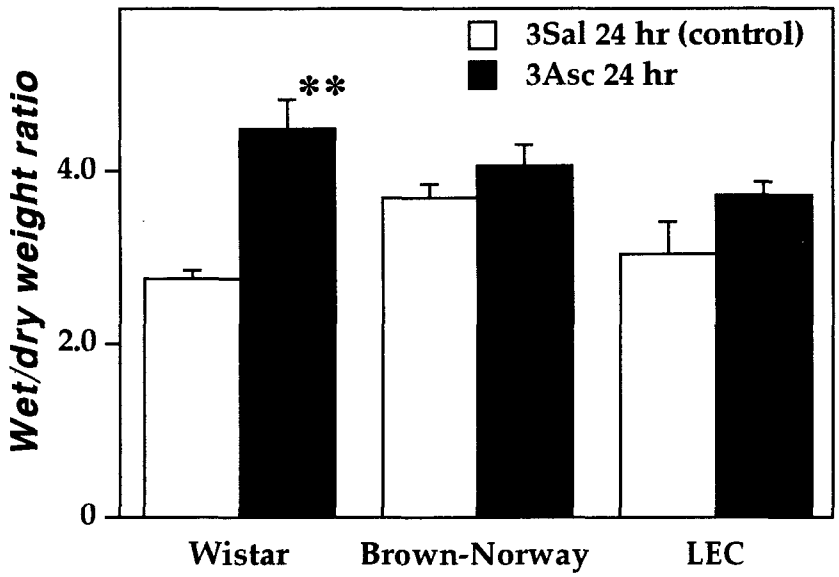


Figure 2-10

The strain differences in the wet to dry (wet/dry) weight ratio of the bronchial tissue isolated from 3 times repeatedly challenged rats with DNP-*Ascaris* antigen (3Asc 24 hr) or from 3 times saline inhaled rats that had been sensitized (3Sal 24 hr; control). Wistar (left), Brown-Norway (middle) and Long-Evans Cinnamon (LEC; right) rats were used. Twenty-four hours after the last challenge, the tissue wet weight of the main bronchial ring was determined and then the tissue was freeze-dried for 24 hr. Then the wet/dry weight ratio was calculated. **$p<0.01$ vs. respective control group.

Experiment 2-4: Strain differences in the generation of antibodies expressed as 48 hr passive cutaneous anaphylaxis titer.

Materials and methods

Sensitization and antigenic challenge

Male Wistar, Brown-Norway (BN) and Long-Evans Cinnamon (LEC) rats were sensitized and received repeated antigenic challenge by the method described in **Experiment 2-1**.

Passive cutaneous anaphylaxis (PCA)

To measure 48 hr PCA titer, the blood specimens were obtained from the heart after the *in vivo* study under anesthesia as described above. The blood specimens were ice-cooled for 10 min and then centrifuged (3000 rpm, 4 ℃ for 10 min). The sera obtained were stored at -35 ℃ until used.

Serial dilutions (4-1024) of the serum obtained from individual bleedings were injected intradermally in 0.05 ml quantities into the shaved backs of normal rats of each strain. After 48 hr, the rats were i.v. injected DNP-Asc (2 mg protein/200 g body weight) together with 1 % E.B. (0.5 ml/200 g body weight) into the tail veins. They were exsanguinated from the abdominal aorta 30 min later, and the skin was reflected for measurement of the extent of extravasation of the dye. When the diameter was ≥5 mm, the dilution was determined as the end point.

Drugs

Evans blue was purchased from Merck (New Jersey, USA).

Statistical analyses

The PCA titer of each group is expressed as the mean and its statistical significance was determined by Wilcoxon rank-sum test.

Results

The antibody titer was measured by 48 hr passive cutaneous anaphylaxis (PCA), 24 hr after the last antigenic challenge or last saline inhalation in sensitized control group. In the respective strain of rats, no significant difference was observed between the sensitized control groups and repeated challenged groups (Table 2-1). On the other hand, the mean PCA titer of

Wistar rats that were subject to repeated antigenic challenge was much and significantly lower than that of BN rats ($p<0.01$), but significantly higher than that of LEC rats ($p<0.05$).

Table 2-1 Strain differences in 48 hr passive cutaneous anaphylaxis (PCA) titers in DNP-Ascaris sensitized or challenged rats.

	Wistar		BN		LEC	
	3Sal	3Asc	3Sal	3Asc	3Sal	3Asc
n	5	6	7	6	6	7
Mean PCA titer	11.2	30.0^{ns}	878**	1024^{ns}**	6.7	6.3^{ns}*
PCA titer range	4-16	4-64	512-1024	1024	4-16	4-16

BN; Brown-Norway rats, LEC; Long-Evans Cinnamon rats, 3Asc; 3 times repeated challenge group, and 3Sal; 3 times repeated saline-received group (only sensitization). Antisera were obtained 24 hr after the last challenge (3Asc) or last saline inhalation (3Sal) ns; no significant difference vs. respective 3Sal group. *$p<0.05$ and **$p<0.01$ vs. respective Wistar group (Wilcoxon rank-sum test).

Discussion

As described in **CHAPTER 1**, repeated antigenic challenge caused a distinct airway hyperresponsiveness (AHR) and airway inflammation both at *in vivo* and *in vitro* levels by using Wistar strain of rats. The AHR and airway inflammation caused appeared severest 24 hr after the last antigenic challenge of the 3 times repeated challenge conducted every 48 hr. In the present study, the strain difference of the pathogenesis of AHR was examined in the strains of rats exhibiting immunological deviation as well as Wistar strain.

Brown-Norway (BN) rats have been known as a high IgE responder (Pauwels *et al.*, 1978; Pauwels *et al.*, 1979). From the veiwpoint of some immunological similarity to humans, some investigators have used this strain as models of early (Bellofiore *et al.*, 1987; Du *et al.*, 1992) and late asthmatic responses (Eidelman *et al.*, 1988; Xu *et al.*, 1990), antigen-induced AHR (Bellofiore and Martin, 1988; Elwood *et al.*, 1991) and allergic rhinitis (Takahashi et al., 1990). Elwood et al. (1991) reported that 7 times repeated challenge with ovalbumin (OA) of OA-sensitized BN rats caused AHR to ACh and significant increase in eosinophils and lymphocytes in bronchoalveolar lavage fluid (BALF). Based on their report and high immunoreactivity of the strain, we expected that more marked AHR could be observed in BN rats than in Wistar rats. However, it turned out that our expectation was disappointed. In the present study, the AHR to ACh induced by 3 times repeated antigenic challenge was greater in Wistar rats than in BN rats. On the other hand, it was found that the *in vivo* airway responsiveness to ACh in normal BN rats was significantly larger than that in normal Wistar rats. These findings indicate that BN rats might be a spontaneously hyperreactive strain and that there might possibly have a limitation for inducing further hyperreactivity because of ceiling effect. Bellofiore and Martin (1988) revealed that some degree of desensitization to OA antigen occurred in sensitized BN rats with 6 times repeated antigen inhalation. The mechanism of desensitization, they said, had yet to be resolved. Possibly, the desensitization process might be somewhat participated in our results.

In the present study, the *in vitro* bronchial responsiveness to ACh and to electrical field stimulation (EFS) were both significantly augmented 24 hr after the last antigenic challenge in Wistar rats. On the other hand, in BN rats, no change in the bronchial responsiveness to ACh was observed, although the responsiveness to EFS was significantly augmented 24 hr after the last antigenic challenge. We have confirmed in our experiment that the contractile responses elicited by EFS were completely abolished by tetrodotoxin (10^{-6} M) or atropine (10^{-6} M), indicating that the EFS responses were mediated by neural, especially cholinergic component. These findings therefore suggest that only a prejunctional hyperfunction (*e.g.* increased release of ACh from the nerve endings) might have occurred in BN rats, whereas smooth muscle

hypersensitivity to ACh possively with a prejunctional hyperfunction) might have occurred in Wistar rats after repeated antigenic challenge. BN rats are likely to be useful for investigating some prejunctional changes with the AHR.

Long-Evans Cinnamon (LEC) strain of rats is a new mutant which develops spontaneous hepatitis associated with severe jaundice at the age of approximately 4 months (Yoshida *et al.*, 1987). It was also reported that this strain of rats has a defect of T cell maturation, that is, an arrest of differentiation from $CD4^+8^+$ to $CD4^+8^-$ thymocytes and dysfunction of helper T cells (Agui *et al.*, 1990; Yamada *et al.*, 1991). Generally, the helper T cells are inevitably concerned in the production of immunoglobulin E (IgE) antibodies, so that the present result representing the lowest PCA titer and minor behavioral change by antigenic challenge in LEC rats is consistent with this notion. Although the mechanisms of pathogenesis of AHR have not yet been disclosed, the interrelationship among the late asthmatic response (LAR), airway inflammation and AHR should be very important (O'Byrne, 1986; Chung, 1986). There is a severe eosinophil infiltration into the airways at LAR (De Monchy *et al.*, 1985; Hutson *et al.*, 1988). There is also increasing evidence that the infiltrating eosinophils cause airway tissue damage and LAR by releasing cytotoxic granules and membrane products (Frigas and Gleich, 1986; Wegner *et al.*, 1990). In addition to the eosinophil infiltration, the recent studies have shown that $CD4^+$ T cells increase in the airways at LAR (Metzger *et al.*, 1987; Frew *et al.*, 1990), suggesting that $CD4^+$ T cells might be involved in antigen-induced LAR and AHR. More recently, Nakajima *et al.* (1992) have demonstrated that the *in vivo* depletion of $CD4^+$ T cells by pretreatment with anti-L3T4 (CD4) monoclonal antibody significantly decreased the eosinophil infiltration into the trachea induced by antigen (OA) inhalation to sensitized mice. These findings indicate that T lymphocytes, especially $CD4^+$ T cells (generally, a marker of helper T cells), may have an important role in pathogenesis of antigen-induced AHR that generally follows LAR. On the other hand, our findings of the failure in enhancement of the airway responsiveness to ACh (*in vivo* and *in vitro*) and electrical field stimulation (*in vitro*) in LEC rats after repeated antigenic challenge, may be presumably due to incomplete sensitization formed, through the defect of T cell maturation.

In the present study, we determined the bronchial tissue wet to dry (wet/dry) weight ratio as an index of airway edema. As a result, a distinct airway inflammation occurred after repeated antigenic challenge in Wistar rats. This observation corresponded with our previous study (Misawa and Chiba, 1993) where the Evans blue method and the histological examination were used. In BN and LEC rats, however, only insignificant increase in the wet/dry weight ratio was observed. We previously demonstrated (Misawa and Chiba, 1993) that the time courses of both the Evans blue exudation and the AHR to ACh after repeated

antigenic challenge resembled, indicating a close relationship between the airway inflammation and AHR. So the strain difference of the pathogenesis of the AHR observed among the three strains of rats, might be reflected by the strain difference of the extent airway edema; Wistar strain of rats which showed a significant airway inflammation developed the AHR.

In our result, the 48 hr PCA titer in BN rats was much higher than that in Wistar rats or LEC rats; the titer in Wistar rats was significantly higher than that in LEC rats. Pauwels et al. (1979) substantiated that there is a genetic influence in the IgE antibody response and that BN strain of rats was the highest IgE responder among the five inbred strains (BN, Wistar R/A, DA, LEW and PVG/C rats) they used. Our result on BN strain was consistent with their findings. Although there had no documentation on the ability of IgE antibody formation in LEC rats, the present result could be explained by their dysfunction of helper T cells as described above. Interestingly, no correlation was observed between the PCA titer and the extent of the AHR caused. The reason why the pathogenesis of the AHR disagree with the extent of the PCA titer in Wistar and BN strains is unknown. BN rats had a high responsivity to ACh both *in vivo* and *in vitro* as described in the result, so that there might have a limitation for inducing further hyperreactivity in BN rats. As another possibility, there may be some functional difference in the mechanism for the AHR induction after the process of IgE production. Wistar strain of rats may be therefore a suitable strain for the formation of antigen-induced AHR.

Presently, we confirmed that the repeated antigenic challenge induced a distinct AHR, both *in vivo* and *in vitro*, and airway inflammation in Wistar rats that were sensitized with DNP-Asc antigen. Some degree of *in vivo* but not *in vitro* ACh hyperresponsiveness was observed in BN rats, but no significant change in both *in vivo* and *in vitro* ACh responsiveness was observed in LEC rats. It has been reported that an antigenic challenge to sensitized guinea pigs (Ishida *et al.*, 1990) and rabbits (Tanaka *et al.*, 1991) resulted in an airway smooth muscle hyperresponsiveness *in vitro*. These animals also obtained AHR in the *in vivo* level (Ishida *et al.*, 1989; Tanaka *et al.*, 1991). These findings therefore suggest that the increased airway smooth muscle responsiveness itself may be reflected in the *in vivo* AHR as a mechanism. In fact, some studies have demonstrated a substantial increase in muscle responsiveness of isolated airway smooth muscle strips from asthmatics *in vitro* (Schellenberg and Foster, 1984; De Jongste *et al.*, 1987; Bai, 1990). In conclusion, Wistar rats may be most suitable for the model of antigen-induced AHR among the strains used.

CHAPTER 3:
The mediators involved in the pathogenesis of antigen-induced airway hyperresponsiveness in rats

Introduction

Thromboxane A_2 (TXA_2) is a potent constrictor of bronchial smooth muscle that is formed after activation of platelets, macrophages and other cells (Svensson *et al.*, 1977; Ullrich & Nusing, 1990).The role that TXA_2 plays in bronchial asthma can be probed by specific inhibitors of the thromboxane synthase enzyme. For example, inhibitors of thromboxane synthase have decreased the bronchoconstrictor response to allergen inhalation in guinea pigs, presumably because of the blockade of TXA_2 formation (Nambu *et al.*, 1990). As for AHR, it was recently reported that TXA_2 synthase inhibitors prevent AHR induced by ozone, platelet activating factor (PAF) and antigen in dogs (Aizawa *et al.*, 1985; Chung *et al.*, 1986a; 1986b) and sheep (Abraham *et al.*, 1988). These findings suggest that TXA_2, generated from inflammatory cells and/or airway tissues, may play an important role in the pathogenesis of AHR. On the other hand, PAF is a potent mediator of inflammation that has been implicated in the pathogenesis of asthma (Barnes *et al.*, 1988), and it has been proposed that PAF is an important mediator in the exacerbation of asthma (Page & Morley, 1986; Barnes & Chung, 1987). It has also been reported that PAF receptor antagonists inhibit AHR induced by antigen in guinea pigs (Coyle *et al.*, 1988; Havill *et al.*, 1990).

In the present study, the effects of a TXA_2 synthase inhibitor, ozagrel, and a PAF antagonist, CV-3988, on the antigen-induced AHR were investigated by using our rat AHR model.

Experiment 3-1: Effect of a platelet activating factor antagonist on antigen-induced airway hyperresponsiveness.

Materials and methods

Animals

Male Wistar rats (6 weeks of age, specific-pathogen-free, 170-190 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water.

Sensitization and antigenic challenge

The method for inducing AHR was described in **Experiment 1-2**. Animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract (DNP-Asc, 2 mg protein, s.c.) together with *Bordetella pertussis* ($2X10^{10}$) as an adjuvant and were boosted by DNP-Asc (0.5 mg protein, i.m.) 5 days later, according to the method of Tada and Okumura (1971). Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc (6 mg protein/ml, 5-6 ml) with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo) for 20 min under unanesthesia in a plexiglass box (300x200 mm, height: 150 mm). Then the animals were subjected to 3 times repeated antigenic challenge every 48 hr with the same inhalational challenge method described above. The sensitized control rats received the same immunization, but were inhaled saline instead of DNP-Asc.

Drug treatments

CV-3988 (3 mg/kg) was intravenously administered from a tail vein 5 min before each antigenic challenge. The doses of CV-3988 used in this study was known to selectively antagonize the PAF-induced response (Misawa & Iwamura, 1990).

Measurement of bronchomotor response

The determination of airway responsiveness to ACh was done 24 hr after the last antigenic challenge. Rats were anesthetized with urethane (2 g/kg, i.p.), placed in the supine position and ventilated artificially through a tracheal cannula at a frequency of 70 breaths/min. Respiratory volume was adjusted at the beginning of the experiment so that ventilation overflow (VO) was 0.5 ml in each rat. Spontaneous respiration was stopped by pancuronium bromide (1 mg/kg, i.v.). The bronchomotor tone was measured by the modified Konzett-Rös sler method (Konzett & Rössler, 1940) as described in **Experiment 1-1**. The lung was

inflated at a fixed volume of air under a constant pressure (5 cmH_2O), and VO was continuously recorded with a combination of pneumotachograph (TP-602T, Nihon Kohden) and integrator (EI-601G, Nihon Kohden). Systemic blood pressure was monitored with a pressure transducer (DX-300, Nihon Kohden) from a cannula inserted into the left carotid artery. All the above parameters were recorded on a polygraph (RM-6000, Nihon Kohden).

ACh solutions (0.001-0.03 %) were cumulatively inhaled each for 3 min to rats by aerosolizing the solution contained in a specially devised plastic cylindrical chamber (capacity: 25 ml) which was introduced in an ultrasonic nebulizer (TUR-3200, Nihon Kohden). The ultrasonic nebulizer with the plastic chamber was placed into the respiratory circuit for the aerosolized mist being inhaled into the airways at each time of ventilation.

Drugs

The following drugs were used: CV-3988 (*(RS)*-2-methoxy-3-(octadecylcalbamoyloxy)-propyl-2-(3-thiazolo)ethyl phosphate; Takeda Pharmaceutical Co., Osaka, Japan); 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane (Sigma, St. Louis, USA).

CV-3988 was dissolved in saline. The other drugs used in the present study were dissolved and/or diluted in saline.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA). The potency ratio of the *in vivo* bronchoconstrictor response to ACh was calculated by parallel line assay.

Results

Inhalation of four cumulative aerosols of ACh (0.001, 0.003, 0.01 and 0.03 %; each for 3 min) to the anesthetized control rats (that were sensitized and inhaled saline instead of DNP-Asc, and pretreated with respective vehicle before each saline inhalation; Veh-3Sal groups) induced a concentration-related increase in ventilation overflow (V.O.), that is, a bronchoconstriction (Fig. 3-1). Pretreatment with CV-3988 (3 mg/kg, *i.v.*) alone had no significant effect on the airway responsiveness to inhaled ACh (data not shown).

In the repeatedly antigenic challenged rats that were pretreated with respective vehicle (Veh-3Asc groups), the concentration-response curve to inhaled ACh was markedly shifted to

he left 24 hr after the last antigenic challenge (9.5 times; its 95 % confidence interval: 4.5-36.9, $F_{[1,40]}=56.6$, $p<0.01$ (in Fig. 3-1)) as compared with Veh-3Sal group. Pretreatment with CV-3988 (3 mg/kg, i.v.) had no significant effect on the increased airway responsiveness after repeated antigenic challenge (Fig. 3-1).

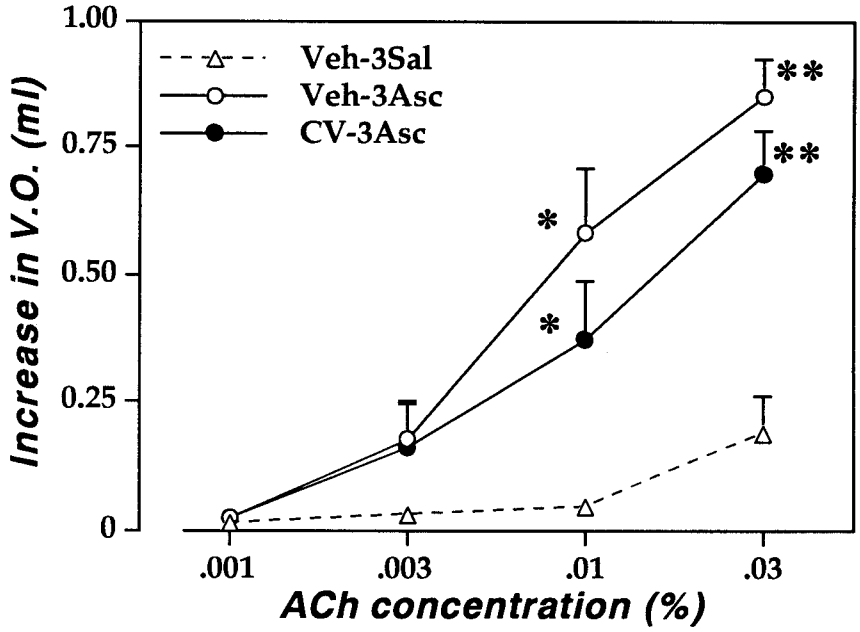


Figure 3-1

The effect of CV-3988 (3 mg/kg, *i.v.*) on airway hyperresponsiveness (AHR) to inhaled acetylcholine (ACh) induced by repeated antigenic challenge in rats. Δ; Animals that were sensitized and inhaled saline 3 times instead of DNP-Asc antigen, and pretreated with saline (vehicle for CV-3988, 0.1 ml/100 g wt, *i.v.*) 5 min before each saline inhalation (n=5). O; Animals that were sensitized and challenged 3 times with DNP-Asc, and pretreated with saline 5 min before each challenge (n=5). ●; Animals that were sensitized and challenged 3 times with DNP-Asc, and pretreated with CV-3988 5 min before each challenge (n=6). Each point represents the mean with S.E. *$p<0.05$ and **$p<0.01$ vs. Veh-3Sal group (unpaired Student's t-test or Cochran-Cox test).

Experiment 3-2: Effect of a thromboxane A_2 synthase inhibitor on antigen-induced airway hyperresponsiveness.

Materials and methods

Sensitization and antigenic challenge

Animals were sensitized and received repeated antigenic challenge by the method described in **Experiment 3-1**.

Drug treatments

Ozagrel (100 mg/kg, *p.o.*) was administered 30 min before each antigenic challenge. The doses of ozagrel used in this study was known to inhibit thromboxane generation (Takehana *et al.*, 1990).

Measurement of bronchomotor response

The determination of airway responsiveness to ACh was measured 24hr after the last antigenic challenge by the method described in **Experiment 3-1**.

Drugs

The following drugs were used: ozagrel hydrochloride ((*E*)-3-[*p*-(1*H*-imidazol-1-ylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate; Kissei Pharmaceutical Co., Matsumoto, Japan); 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane (Sigma, St. Louis, USA).

Ozagrel was dissolved in water. The other drugs used in the present study were dissolved and/or diluted in saline.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA). The potency ratio of the *in vivo* bronchoconstrictor response to ACh was calculated by parallel line assay.

Results

Inhalation of four cumulative aerosols of ACh (0.001, 0.003, 0.01 and 0.03 %; each

for 3 min) to the anesthetized control rats (that were sensitized and inhaled saline instead of DNP-Asc, and pretreated with respective vehicle before each saline inhalation; Veh-3Sal groups) induced a concentration-related increase in ventilation overflow (V.O.), that is, a bronchoconstriction (Fig. 3-2). Pretreatment with ozagrel (100 mg/kg, *p.o.*) alone had no significant effect on the airway responsiveness to inhaled ACh (data not shown).

In the repeatedly antigenic challenged rats that were pretreated with vehicle (Veh-3Asc groups), the concentration-response curve to inhaled ACh was markedly shifted to the left 24 hr after the last antigenic challenge (5.5 times; its 95 % confidence interval: 3.4-11.0, $F_{[1,40]}=55.2$, $p<0.01$ (in Fig. 3-2)) as compared with Veh-3Sal group. The ACh hyperresponsiveness after repeated antigenic challenge was significantly attenuated by pretreatment with ozagrel (100 mg/kg, *p.o.*) before each challenge ($F_{[1,45]}=8.68$, $p<0.01$). The airway responsiveness to inhaled ACh was significantly inhibited at a concentration of 0.03 % ($p<0.05$) as compared with Veh-3Asc group. However, this inhibition was only partial: the ACh responsiveness was still significantly higher than Veh-3Sal group at concentrations of 0.01 and 0.03 % ($p<0.05$, Fig. 3-2).

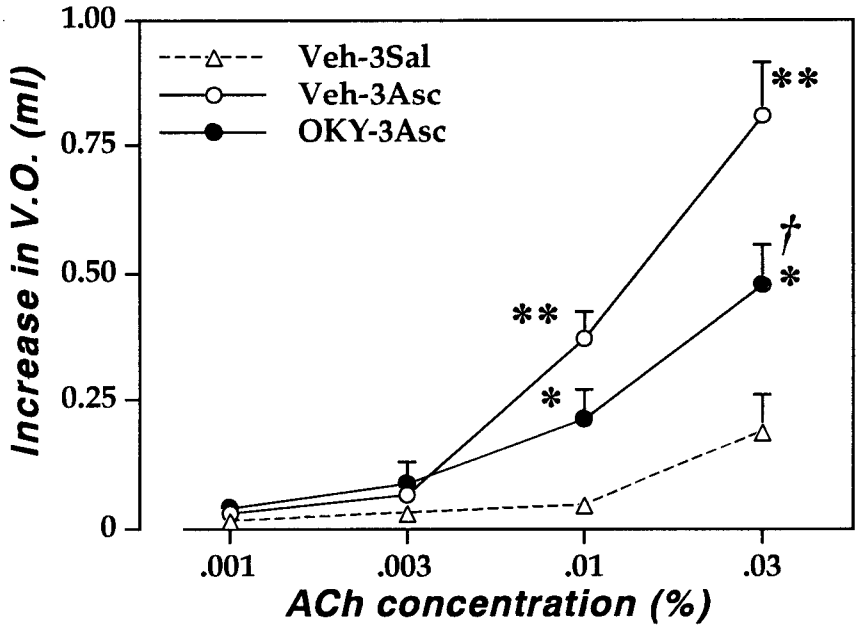


Figure 3-2

The effect of ozagrel (100 mg/kg, *p.o.*) on airway hyperresponsiveness (AHR) to inhaled acetylcholine (ACh) induced by repeated antigenic challenge in rats. Δ; Animals that were sensitized and inhaled saline 3 times instead of DNP-Asc antigen, and pretreated with water (vehicle for ozagrel, 0.2 ml/100 g wt, *p.o.*) 30 min before each saline inhalation (n=5). O; Animals that were sensitized and challenged 3 times with DNP-Asc, and pretreated with water 30 min before each challenge (n=5). ●; Animals that were sensitized and challenged 3 times with DNP-Asc, and pretreated with ozagrel 30 min before each challenge (n=6). Each point represents the mean with S.E. *$p<0.05$ and **$p<0.01$ vs. Veh-3Sal group and †$p<0.05$ vs. Veh-3Asc group (unpaired Student's t-test or Cochran-Cox test).

Discussion

In the present study, the airway hyperresponsiveness (AHR) to inhaled acetylcholine (ACh) induced by repeated antigenic challenge was significantly attenuated by the pretreatment with a thromboxane A_2 (TXA_2) synthase inhibitor, ozagrel (100 mg/kg, *p.o.*), 30 min before each antigenic challenge. This finding suggests an important role of TXA_2 in the pathogenesis of antigen-induced AHR in rats. On the other hand, a platelet activating factor (PAF) receptor antagonist, CV-3988, had no effect in our rat AHR.

It has been well known that TXA_2 is generated and released by various stimuli from inflammatory cells (*e.g.* mast cells, eosinophils) and airway tissue. U-46619, a stable thromboxane mimetic, reportedly induced tracheal contraction (Nagai *et al.*, 1991) and potentiated the cholinergic contractile response induced by field stimulation (Chung *et al.*, 1985). These findings suggest that the released TXA_2 modulates the airway tone. Furthermore, Aizawa *et al* (1985) reported that the AHR to inhaled ACh induced by ozone exposure was inhibited by ozagrel, and that inhalation of subthreshold concentration of U-46619, which did not cause any bronchoconstriction, increased airway responsiveness to inhaled ACh in dogs. Similarly, it was also reported that ozagrel prevented the AHR to inhaled ACh induced by antigenic challenge in dogs (Chung *et al.*, 1986a) and in guinea pigs (Komatsu *et al.*, 1990). These observations and our present result suggest that TXA_2 might have a key role on the pathogenesis of the AHR. Komatsu *et al* (1990) reported that the antigen-induced AHR was completely abolished by pretreatment with 100 mg/kg of ozagrel (the same dosage in the present study), although only partial inhibitory effect was observed in our study. The species differences (guinea pigs *vs.* rats) and/or the differences of drug administration routes (intraduodenally *vs.* oral administration) might be involved in the controversy, but it is probable that other mechanism(s) might be contribute to the pathogenesis of the AHR in our rat model.

Although the source of TXA_2 was not determined in the present study, the mast cells and polymorphonuclear leukocytes might be involved in, as the following reasons; first, we previously demonstrated that this rat AHR was inhibited by treatment with anti-allergic drugs (Misawa *et al.*, 1991) and it was reported that the IgE-dependent allergic bronchoconstriction was inhibited by ozagrel in rats (Watanabe & Hayashi, 1990). Secondly, we also demonstrated the inflammatory cells (*e.g.* eosinophils) infiltration to airway tissues at antigen-induced AHR in rats (**CHAPTER 1**). Furthermore, it was reported that the allergen-induced late phase bronchoconstriction, probably caused by inflammatory cells and resulted in the AHR, was inhibited by ozagrel in rats (Watanabe & Hayashi, 1990).

PAF is also known to be released from inflammatory cells including eosinophils (Lee *et*

al., 1984) and neutrophils (Clark *et al.*, 1980) *etc.* and is suggested to cause antigen-induced AHR in guinea pigs (Coyle *et al.*, 1988; Havill *et al.*, 1990). However, Chung *et al* (1986b) reported that AHR induced by PAF was inhibited by pretreatment with ozagrel in dogs. On the other hand, it was reported that the structurally unrelated PAF antagonists, CV-3988 and L-652,731, had no effect on the anaphylactic bronchoconstriction although these antagonists inhibited the PAF-induced bronchoconstriction in guinea pigs (Danko *et al.*, 1988). These findings suggest that PAF would not be a final and/or important mediator involved in the asthmatic reaction and/or the pathogenesis of the AHR, although PAF has a potency to cause bronchoconstriction (Danko *et al.*, 1988; Misawa & Takata, 1988) and AHR (Chung *et al.*, 1986b).

Our results demonstrated that TXA_2, but not PAF, is one of the most important mediators participating in the pathogenesis of the antigen-induced AHR in rats. However, other mechanism(s) might be implicated in this AHR because the inhibition by ozagrel was only partial.

CHAPTER 4:
Participation of sensory neuropeptides in the pathogenesis of antigen-induced airway hyperresponsiveness in rats

Introduction

Our previous study showed that repeated antigenic challenge, but not sensitization alone, did cause both marked AHR and airway inflammation (increased permeability and inflammatory cells infiltration) in rats (**CHAPTER 1**). There has been considerable interest in the possible involvement of sensory neuropeptides, such as substance P (SP) and neurokinin A (NKA), in the airway inflammation, and more recent studies suggest the importance of sensory neuropeptides in the pathogenesis of AHR induced by cigarette smoke (Daffonchio *et al.*, 1990), toluene diisocyanate (Thompson *et al.*, 1987) and antigen (Matsuse *et al.*, 1991) in guinea pigs. However, in these studies, the exact mechanism by which sensory neuropeptides induce AHR has yet to be resolved. These peptides are localized at unmyelinated C-fiber afferents and have been considered to play an important role in the direct (Sekizawa *et al.*, 1987) and/or indirect (Hall *et al.*, 1989; Tanaka & Grunstein, 1986) regulation of tracheobronchial smooth muscle tone. Capsaicin, the pungent agent of hot peppers, is well known to selectively release and deplete such neuropeptides, and is frequently used as a tool of physiological and/or pharmacological studies. Therefore, in the present study, at first, the effect of sensory neuropeptides depletion by systemic capsaicin procedure on the pathogenesis of our antigen-induced AHR in rats was investigated.

It is well known that these neuropeptides are hydrolyzed and inactivated by neutral endopeptidase (NEP; EC 3.4.24.11; also called enkephalinase), a membrane-bound Zn-metallopeptidase localized richly at airway epithelial and smooth muscle cells. Recently, the NEP hypoactivity was reported at the AHR induced by ozone (Murlas *et al.*, 1992) and acrolein, an aldehyde component of cigarette smoke and smog (Turner *et al.*, 1993) in guinea pigs. These findings suggest that an NEP hypoactive state itself may be involved in the AHR, including the antigen-induced AHR. We therefore examined the effects of the NEP inhibitor, phosphoramidon, on the airway responsiveness of our repeated antigenic challenge-induced airway hyperresponsive rats. Finally, we directly measured the NEP activity of airway tissues obtained from the repeatedly antigen-challenged rats which showed AHR.

Experiment 4-1: Effect of sensory neuropeptides depletion on antigen-induced airway hyperresponsiveness.

Materials and methods

Animals

Male Wistar rats (6 weeks of age, specific-pathogen-free, 170-190 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water.

Sensitization and antigenic challenge

The method for inducing AHR was described in **Experiment 1-2**. Animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract (DNP-Asc, 2 mg protein, s.c.) together with *Bordetella pertussis* ($2X10^{10}$) as an adjuvant and were boosted by DNP-Asc (0.5 mg protein, i.m.) 5 days later, according to the method of Tada and Okumura (1971). Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc (6 mg protein/ml, 5-6 ml) with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo) for 20 min under unanesthesia in a plexiglass box (300X200 mm, height: 150 mm). Then the animals were subjected to 3 times repeated antigenic challenge every 48 hr with the same inhalational challenge method described above. The sensitized control rats received the same immunization, but were inhaled saline instead of DNP-Asc.

In vivo study

The determination of airway responsiveness to ACh was done 24 hr after the last antigenic challenge. Rats were anesthetized with urethane (2 g/kg, i.p.), placed in the supine position and ventilated artificially through a tracheal cannula at a frequency of 70 breaths/min. Respiratory volume was adjusted at the beginning of the experiment so that ventilation overflow (VO) was 0.5 ml in each rat. Spontaneous respiration was stopped by pancuronium bromide (1 mg/kg, i.v.). The bronchomotor tone was measured by the modified Konzett-Rös sler method (Konzett & Rössler, 1940) as described in **Experiment 1-1**. The lung was inflated at a fixed volume of air under a constant pressure (5 cmH_2O), and VO was continuously recorded with a combination of pneumotachograph (TP-602T, Nihon Kohden) and integrator (EI-601G, Nihon Kohden). Systemic blood pressure was monitored with a pressure transducer (DX-300, Nihon Kohden) from a cannula inserted into the left carotid artery. All the above parameters were recorded on a polygraph (RM-6000, Nihon Kohden).

ACh solutions (0.001-0.03 %) were cumulatively inhaled each for 3 min to rats by aerosolizing the solution contained in a specially devised plastic cylindrical chamber (capacity: 25 ml) which was introduced in an ultrasonic nebulizer (TUR-3200, Nihon Kohden). The ultrasonic nebulizer with the plastic chamber was placed into the respiratory circuit for the aerosolized mist being inhaled into the airways at each time of ventilation.

In some experiments, the neutral endopeptidase (NEP) inhibitor, phosphoramidon (dissolved in saline) was intravenously administered (3 mg/kg) 5 min before the determination of airway responsiveness to ACh. This dosage of phosphoramidon had no effects on basal bronchomotor tone and systemic blood pressure, but in normal nonsensitized rats, the airway responsiveness to inhaled ACh was markedly increased after phosphoramidon treatment (see **CHAPTER 5**).

Systemic capsaicin pretreatment. The capsaicin treatment was done by using the method of Yonei *et al.* (1990) with a minor modification. Animals were treated with a total dose of 125 mg/kg capsaicin, given subcutaneously in two increasing doses over 3 days, 10 days before starting repeated antigenic challenge. Each dose was given as follows: Day 1, 25 mg/kg; Days 2 and 3, 50 mg/kg of capsaicin. Capsaicin was dissolved in 10 % ethanol-10 % Tween 80-80 % saline. To counteract respiratory impairment caused by capsaicin, terbutaline (1 mg/kg, s.c., 60 min before) and aminophylline (25 mg/kg, i.p., 30 min before) was pretreated before each capsaicin treatment under anesthetization with ketamine (50 mg/kg, s.c.) and thiopental (40 mg/kg, i.p.). The same dosage regimen, including anesthetization, was used in control animals to deliver an equivalent volume of vehicle (10 % ethanol and 10 % Tween 80 in saline).

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); capsaicin (Wako Pure Chemical Industries, Osaka, Japan); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane (Sigma, St. Louis, USA).

The drugs used in the present studies were dissolved and/or diluted in saline.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA). The potency ratio of the *in vivo* bronchoconstrictor response to ACh was calculated by parallel line assay.

Results

The airway responsiveness to inhaled ACh was markedly increased 24 hr after the last repeated antigenic challenge (Figs. 4-1). This antigen-induced AHR was significantly attenuated by systemic capsaicin pretreatment; the ACh concentration-response curve of capsaicin-treated group was significantly shifted to the right (3.2 times; its 95 % confidence interval: 2.0-4.9, $p<0.05$) as compared with that of vehicle-treated group (Fig. 4-1).

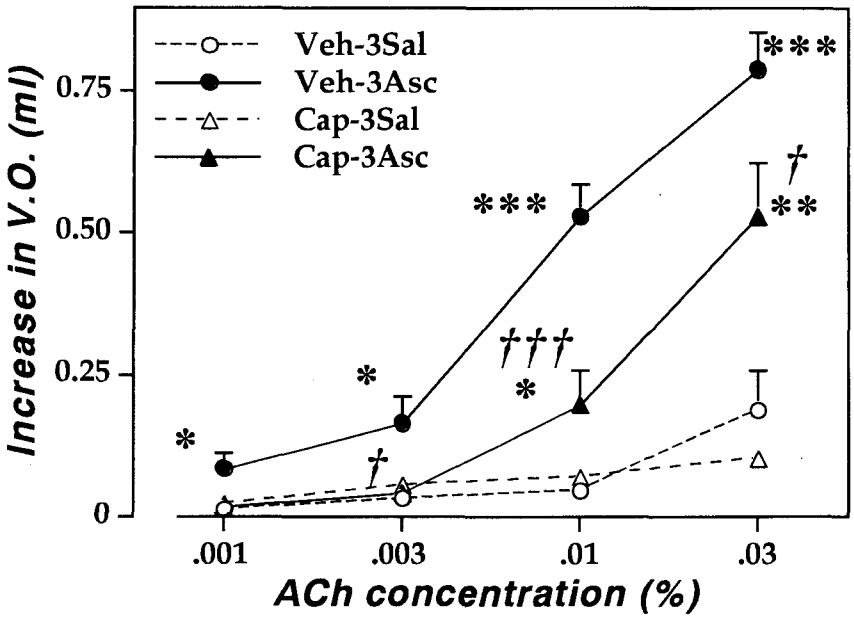


Figure 4-1

The effect of systemic capsaicin pretreatment on the airway hyperresponsiveness to ACh induced by repeated antigenic challenge in rats. Animals were sensitized and inhaled saline (Sal; O, △) or DNP-*Ascaris* antigen (Asc; ●, ▲) 3 times after pretreatment with capsaicin (Cap; △, ▲) or vehicle (Veh; O, ●) for 3 days. Capsaicin (total dose; 1 mg/kg, *s.c.*) was dissolved in 10 % Tween 80 - 10 % ethanol in saline. Values are means ± S.E. from 5 to 14 animals. *p<0.05, **p<0.01 and ***p<0.001 vs. Veh-3Sal group. †p<0.05 and †††p<0.001 vs. Veh-3Asc group.

Experiment 4-2: Effect of phosphoramidon on airway responsiveness after repeated antigenic challenge.

Materials and methods

Sensitization and antigenic challenge

Animals were sensitized and received repeated antigenic challenge by the method described in **Experiment 4-1**.

In vivo study

The determination of airway responsiveness to ACh was measured 24hr after the last antigenic challenge by the method described in **Experiment 4-1**.

The neutral endopeptidase (NEP) inhibitor, phosphoramidon (dissolved in saline) was intravenously administered (3 mg/kg) 5 min before the determination of airway responsiveness to ACh. This dosage of phosphoramidon had no effects on basal bronchomotor tone and systemic blood pressure, but in normal nonsensitized rats, the airway responsiveness to inhaled ACh was markedly increased after phosphoramidon treatment (unpublished data).

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane, phosphoramidon (Sigma, St. Louis, USA).

The drugs used in the present studies were dissolved and/or diluted in saline.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA). The potency ratio of the *in vivo* bronchoconstrictor response to ACh was calculated by parallel line assay.

Results

Figure 4-2 shows the *in vivo* effect of pretreatment with phosphoramidon on the airway responsiveness to ACh in the antigen-induced AHR rats. The phosphoramidon-induced AHR was no more observed in repeatedly antigen-challenged rats unlike in normal rats, indicating that the NEP activity might already have been reduced after repeated antigenic challenge.

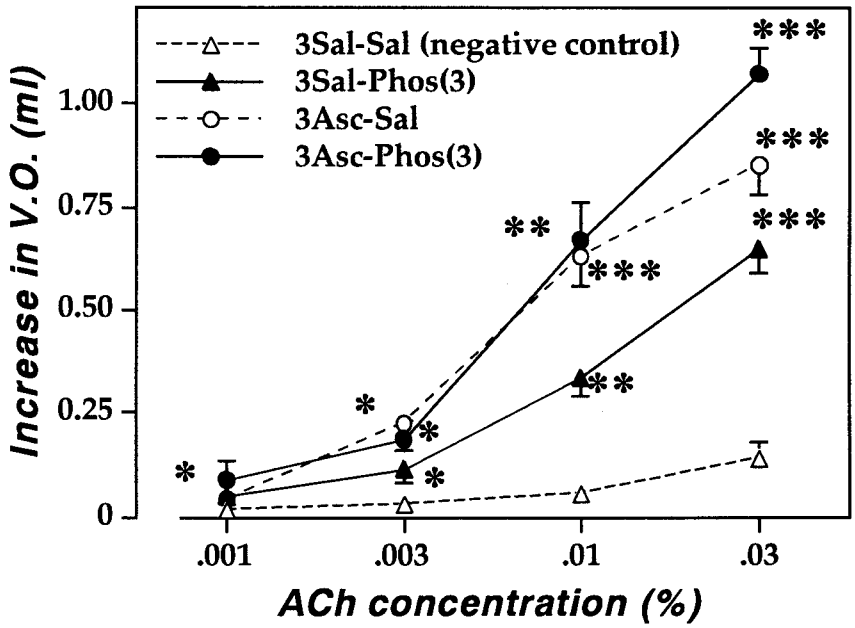


Figure 4-2

The effect of phosphoramidon (Phos) on the repeated antigenic challenge-induced airway hyperresponsiveness to ACh in sensitized rats. Animals were sensitized and inhaled saline (Sal; △, ▲) or DNP-*Ascaris* antigen (Asc; O, ●) 3 times, and then pretreated with Sal (△, O) or Phos (3 mg/kg, *i.v.*; ▲, ●) 5 min before measurement of airway responsiveness to ACh. Values are means ± S.E. from 5 to 6 animals. *p<0.05, **p<0.01 and ***p<0.001 vs. negative control group.

Experiment 4-3: Effect of repeated antigenic challenge on airway tissue neutral endopeptidase activity.

Materials and methods

Sensitization and antigenic challenge

Animals were sensitized and received repeated antigenic challenge by the method described in **Experiment 4-1**.

Assay of NEP activity

Animals were sacrificed by the blow to the head and exsanguinated, and the trachea and bronchus were immediately removed and carefully cleaned of adhering connective tissues. The tracheae, main bronchi and superior lobus of left lungs were collected from 7-9 animals to obtain one preparation for one number of experiment. These tissue segments were homogenized with Physcotron (NITI-ON, Co. Ltd., Japan: 5 sec x 6; max) in 10 vol of ice-cold 0.32 M sucrose. These whole tissue homogenates were centrifuged (1,500 g, 4 ℃ for 15 min) and then the resultant supernatants were recentrifuged (50,000 g, 4 ℃ for 15 min). The resultant pellets were resuspended in 10 vol of ice-cold 50 mM tris(hydroxymethyl)aminomethane (Tris) HCl buffer (pH 7.4) and homogenized (5 sec; max). The suspensions were again centrifuged (50,000 g, 4 ℃ for 15 min) and the pellets were resuspended in 10 vol of ice-cold 50 mM Tris HCl buffer (pH 7.4). These membrane preparations were stored at -80 ℃ until use. The protein concentrations of these preparations were determined by the method of Lowry *et al.* (1951) in triplicate with bovine serum albumin as a standard.

The airway tissue NEP activity assay was performed by using the membrane preparations from normal control, sensitized control and repeatedly antigenic challenged rats, and the results obtained were compared with each other. The NEP activity was determined by the method of Bateman *et al.* (1989) with a minor modification. The hydrolysis of [D-Ala^2,Met^5]enkephalin (Tyr-D-Ala-Gly-Phe-Met) by NEP activity was measured following the appearance of the product Phe-Met. The assay tube (total volume of 100 μl, containing 0.05 mg protein of the membrane preparation) was preincubated with 10^{-5} M captopril and puromycin with/without 10^{-6} M phosphoramidon at 37 ℃. Ten min later, 0.5 mM [D-Ala^2,Met^5]enkephalin was added in the assay tube and incubated at 37 ℃ for 10 min. The reaction was stopped by rapid ice-cooling, and then the suspensions were centrifuged (50,000 g, 4 ℃ for 15 min). The resultant supernatant was directly analyzed using a high-performance

liquid chromatography (HPLC) system (Jasco, Japan) with a CrestPak C18S column (Jasco, Japan). It was eluted at 1 ml/min with 0.02 % trifluoroacetic acid/acetonitrile using a one-step linear gradient consisting of 10-45 % acetonitrile for 12 min. Elutions were monitored at 214 nm. Under these conditions, Phe-Met and [D-Ala^2, Met^5]enkephalin eluted at 5.5±0.0 and 7.8±0.0 min, respectively (Fig. 4-3). Immediately thereafter, acetonitrile was increased to 70 % within 2 min and maintained for the ensuing 5 min to wash off the column before subsequent use. Products detected at 214 nm were identified by coelution with standard. Products were quantitated by interpolating the peak height to a calibration curve generated with authentic standard. All the above assays were performed in duplicate.

We also measured angiotensin converting enzyme (ACE)-like activity of airway tissue preparations. ACE-like activity was calculated by subtracting the generated Phe-Met in the presence of 10^{-5} M captopril (without phosphoramidon) from that in the absence of 10^{-5} M captopril.

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); Tris (Wako Pure Chemical Industries, Osaka, Japan); phosphoramidon, captopril, aminophylline, [D-Ala^2,Met^5]enkephalin, Phe-Met (Sigma, St. Louis, USA).

The drugs used for the NEP activity assay was dissolved and diluted in Tris HCl buffer (pH 7.4).

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

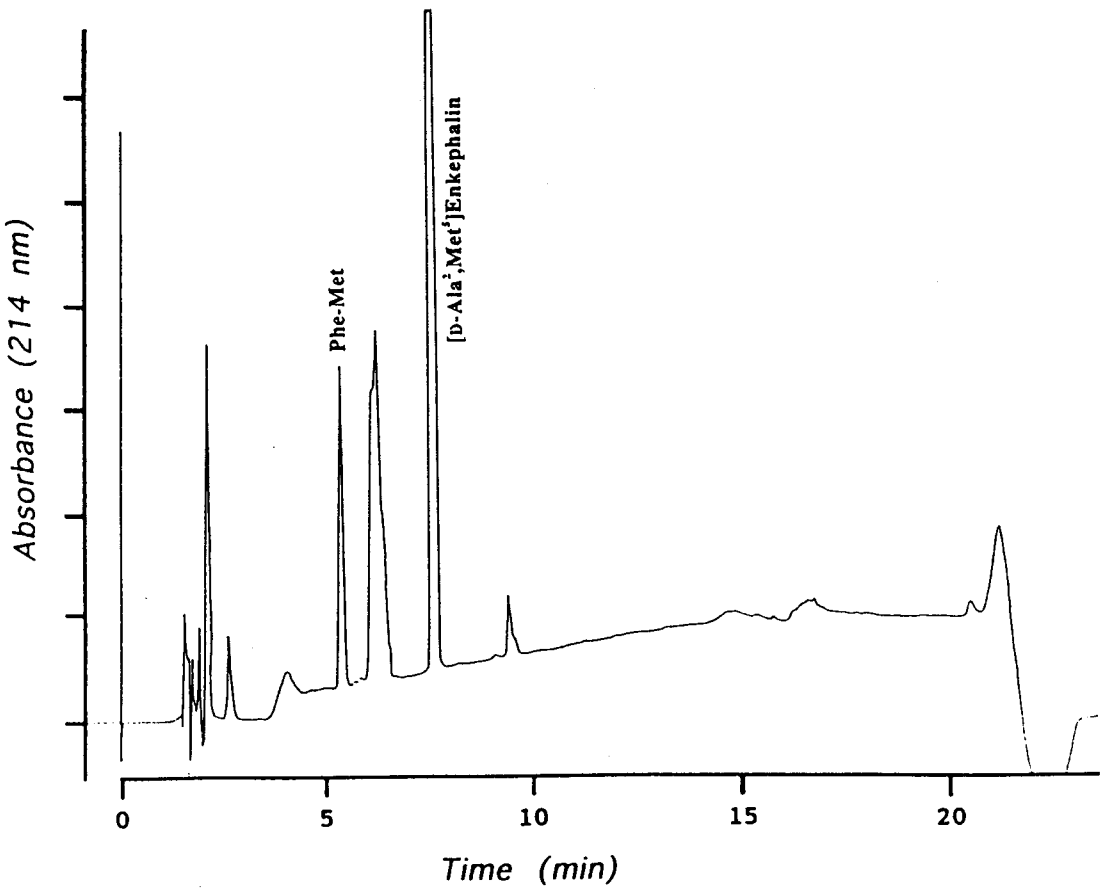


Figure 4-3

Typical reverse-phase, high-pressure liquid chromatography profile of Phe-Met and [D-Ala^2,Met^5]enkephalin recovered from the resultant supernatant that was preincubated with the normal rat airway preparation (*see* **Materials and methods**). The generated Phe-Met (nmol/min/mg protein) was measured as the NEP activity.

Results

Incubation of the airway tissue preparation with [D-Ala^2,Met^5]enkephalin in the presence of captopril and puromycin caused the generation of Phe-Met (Fig. 4-3). The NEP activity, calculated by the generation of Phe-Met, of normal rat airway tissues was 15.6 ± 0.5 nmol/min/mg protein (n=5) (Fig. 4-4). This activity was completely blocked by 10^{-6} M of phosphoramidon (0.0±0.1 nmol/min/mg protein, n=3). The sensitization alone had no effect on the NEP activity (13.8 ± 1.1 nmol/min/mg protein, n=5). However, the NEP activity was significantly reduced 24 hr after the last challenge of 3 times repeated antigenic challenge (9.8 ± 1.2 nmol/min/mg protein, n=5); $p<0.01$ vs. normal group and $p<0.05$ vs. sensitized control group. Figure 4-5 shows the angiotensin-converting enzyme (ACE)-like activity, calculated by subtracting the generated Phe-Met in the presence or absence of 10^{-5} M captopril (without phosphoramidon). No significant difference was observed about the ACE-like activity among the three groups.

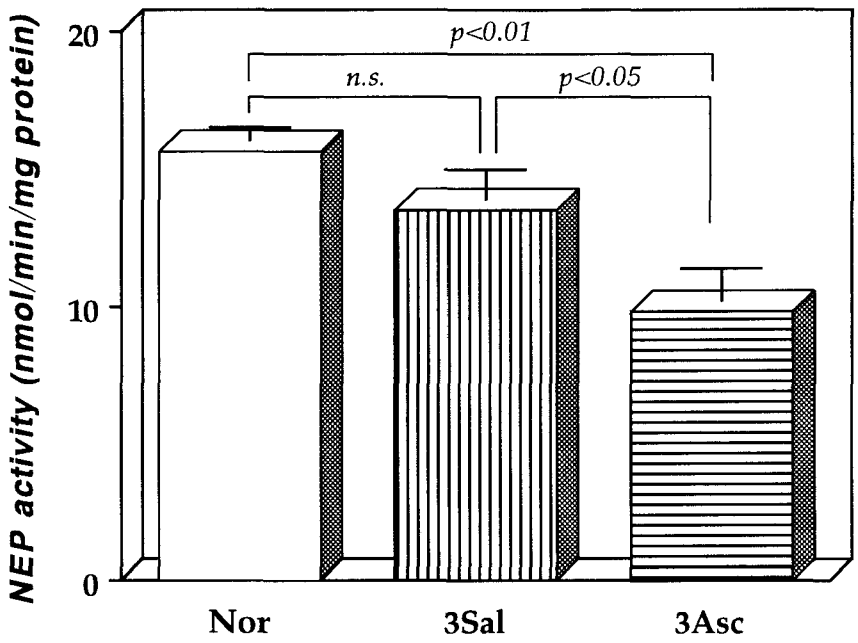


Figure 4-4

Neutral endopeptidase (NEP) activity of airway tissue preparation of normal [Nor; *open column*], sensitized control [3Sal: sensitized and inhaled saline 3 times instead of DNP-*Ascaris* antigen (Asc); *vertically striped column*] and repeated antigen-challenged (3Asc: sensitized and challenged 3 times; *horizontally striped column*) rats. Animals were sacrificed at 24 hr after the last antigenic challenge or the last inhalation of saline, and airway tissues (tracheae, main bronchi and superior lobus of left lungs) were collected from 7-9 animals to obtain one preparation for one experiment. These airway tissues were homogenized, and then NEP activity was measured as described in **Materials and methods**. Values are means ± S.E. from 5 experiments. *n.s.*; no significant difference.

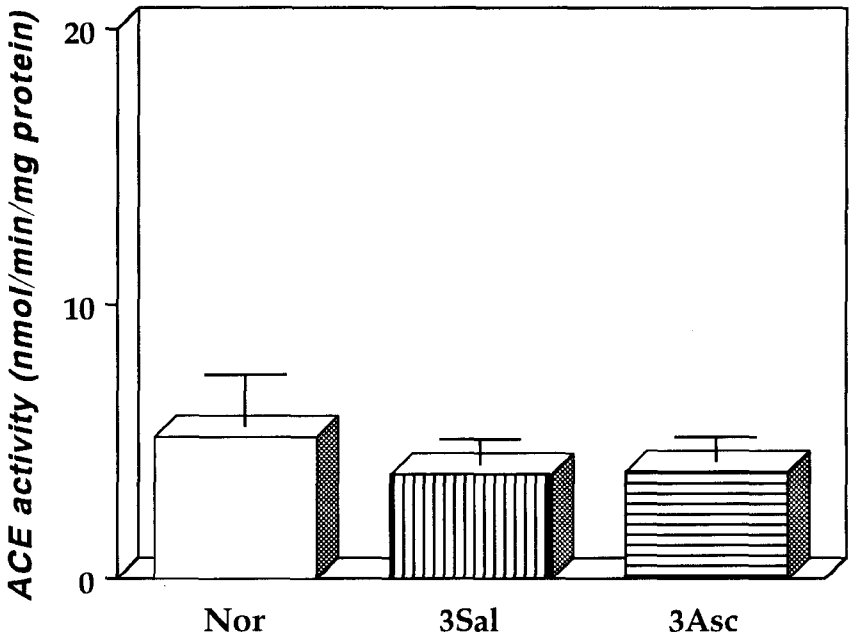


Figure 4-5

Angiotensin converting enzyme (ACE)-like activity of airway tissue preparation of normal [Nor; *open column*], sensitized control [3Sal: sensitized and inhaled saline 3 times instead of DNP-*Ascaris* antigen (Asc); *vertically striped column*] and repeated antigen-challenged (3Asc: sensitized and challenged 3 times; *horizontally striped column*) rats. ACE-like activity was calculated as described in **Materials and methods**. No significant difference was observed about the ACE-like activity among groups.

Discussion

In the present study, the airway hyperresponsiveness (AHR) to inhaled acetylcholine (ACh) induced by repeated antigenic challenge was significantly attenuated by the depletion of sensory neuropeptides by using the systemic capsaicin procedure. This finding suggests an important role of sensory neuropeptides in the pathogenesis of antigen-induced AHR in rats. On the other hand, intravenous administration of a neutral endopeptidase (NEP) inhibitor, phosphoramidon (3 mg/kg) alone, increased airway responsiveness to ACh; the dose of phosphoramidon exerts anti-NEP effect in rats (Umeno *et al.*, 1989; Umeno *et al.*, 1990). This phenomenon suggests a hypothesis that an NEP hypoactive state itself causes an increased airway responsiveness in rats. In support of our hypothesis, the NEP activity was indeed significantly reduced to the extent of about 60 % at the AHR observed after repeated antigenic challenge in sensitized rats as compared with that of normal rats (Fig. 4-4). This is the first study, in our knowledge, that demonstrated the NEP hypoactivity at antigen induced-AHR.

In the present study, the phosphoramidon-induced AHR to inhaled ACh was not observed in the repeatedly antigen-challenged airway hyperresponsive rats (Fig. 4-2), also suggesting a NEP hypoactive state at the antigen induced-AHR. It is well known that NEP is localized rich particularly in the epithelium of the airways (Borson, 1991). Therefore, it could be likely that epithelial cells were denuded after the repeated antigenic challenge. However, the idea would be denied, because we have previously confirmed histologically that epithelial cells were not denuded, but moderately changed after the repeated antigenic challenge (*e.g.*, increased and hypertrophied goblet cells) (see **CHAPTER 1**). Although the mechanism of pathogenesis of the NEP hypoactivity induced by the repeated antigenic challenge was not examined in the present study, as a possible explanation, reactive oxygen species might be involved in this event. It is reported that hypochlorite (HOCl) appears to have a direct effect on the NEP of airway epithelial cells (Lang & Murlas, 1991; Murlas *et al.*, 1990) and enhances the airway responsiveness to SP (Murlas *et al.*, 1990). These findings might support our speculation. It must, however, be taken into consideration that phosphoramidon may affect endothelin converting enzyme (Okada *et al.*, 1990) and NEP modulates the effect of other peptides such as atrial natriuretic peptide (Connell & Jardine, 1990) and endothelin (McKay *et al.*, 1992).

It has been known that sensory neuropeptides, such as NKA and SP, are degraded by not only NEP but also ACE (Shore *et al.*, 1988; Skidgel *et al.*, 1984). We therefore tried to determine the ACE activity. As shown in Fig. 4-5, no significant change was observed about the ACE-like activity after repeated antigenic challenge. It is possible that the difference of the distribution of these enzymes might be reflexed in the results; it has been reported, by means

of immunohistochemical staining, that the ACE activity was localized at the luminal surface of vascular endothelium, whereas NEP activity was localized within airway epithelial and smooth muscle cells (Sekizawa *et al.*, 1987; Johnson *et al.*, 1985). We also have a little discrepancy that the ability of phosphoramidon to increase airway responsiveness was completely abolished in the repeatedly antigenic challenged rats although the NEP activity was reduced by about 40 % in these animals. It is also possible that airway alteration at repeated antigenic challenge-induced AHR might be restricted in cells of airway tissue. A further possible reason would be that the NEP activity was reduced to a much greater extent at the airway epithelia than at other airway tissues, which might result in a great hyperresponsiveness when the antigen was exposed by inhalation. Alternatively, there might be a limitation for inducing further hyperreactivity in repeatedly antigenic challenged rats that acquired severe AHR, in terms of a ceiling effect.

In summary, we have demonstrated that the depletion of neuropeptides by systemic capsaicin treatment in rats significantly attenuates the development of the AHR after repeated antigenic challenge. Furthermore, we also have demonstrated that an NEP hypoactive state in the airways occurs at the antigen-induced AHR in rats. These findings, including our previous study, suggest that repeated antigenic challenge to sensitized rats causes the airway NEP hypoactivity, which in turn results in the sensory neuropeptides accumulation released by various stimuli to cause the AHR. However, other mechanisms may also be responsible for the antigen-induced AHR in rats, because the inhibition of this AHR by systemic capsaicin treatment was partial in our study. Further study is necessary to elucidate the mechanisms of the antigen-induced AHR.

CHAPTER 5:
Effect of neutral endopeptidase inhibition on airway responsiveness to acetylcholine in nonsensitized normal rats

Introduction

The mechanism(s) of airway hyperresponsiveness (AHR) has not been determined. However, there have been reports suggesting a role of neuropeptides in the development of AHR (Ladenius *et al.*, 1991; Matsuse *et al.*, 1991). Neuropeptide-containing nerves have been demonstrated to innervate the airways of several species including guinea pig, man (Lundberg *et al.*, 1984) and rabbit (Black *et al.*, 1992). A variety of chemical, physical or electrical stimuli can cause sensory C-fibers to release neuropeptides locally into the innervated structures, and these released neuropeptides can cause wide variety of physiological events such as bronchoconstriction, bronchial vasodilation, mucus hypersecretion and/or recruitment of inflammatory cells into the airway wall (Barnes, 1991; Solway & Leff, 1991). Several studies (Hooper *et al.*, 1985a; 1985b) have shown that the effects of endogenous tachykinins are limited by neutral endopeptidase (NEP). NEP is a membrane-bound protease that cleaves a large variety of peptidic hormons in the immediate vicinity of their specific receptors. NEP-like activity is specifically found in the epithelium, glands, nerves and smooth muscle cells of tracheobronchial mucosa (Johnson *et al.*, 1985; Ronco *et al.*, 1988). However, there has been poor documentation about NEP activity in rat airway tissues. It has been suggested that inhibition of this enzyme causes the exaggerated actions of both endogenously released and exogenously applied neuropeptides (Sekizawa *et al.*, 1987a; Sekizawa *et al.*, 1987b; Thompson & Sheppard, 1988).

For the purpose of disclosing the mechanism of AHR, we recently developed an animal model of AHR by using Wistar rats that were actively sensitized with dinitrophenylated-*Ascaris suum* antigen (DNP-Asc) and challenged 3 times by inhaling the aerosolized antigen (**CHAPTERS 1** and **2**). In our recent investigation, this antigen-induced AHR was markedly attenuated by the depletion of sensory neuropeptides, by using the systemic capsaicin procedure before sensitization, indicating that endogenous neuropeptides has an important role in inducing the antigen-induced AHR in rats (**CHAPTER 4**). So in the present study, at first, the effects of inhaled NKA and SP in the presence or absence of NEP inhibitors on airway responsiveness to acetylcholine (ACh) were investigated by using nonsensitized

normal rats. Interestingly, in our results, the pretreatment with NEP inhibitors alone induced AHR to inhaled ACh. So next, the mechanism(s) of the NEP inhibitors-induced AHR to ACh were investigated.

Experiment 5-1: Effects of exogenous neuropeptides and neutral endopeptidase inhibitors on *in vivo* airway responsiveness to inhaled acetylcholine.

Materials and methods

Animals

Male Wistar rats (8 weeks of age, specific-pathogen-free, 230-310 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water.

***In vivo* study**

Rats were anesthetized with urethane (2 g/kg, i.p.), placed in the supine position and ventilated artificially through a tracheal cannula at a frequency of 70 breaths/min. Respiratory volume was adjusted at the beginning of the experiment so that ventilation overflow (VO) was 0.5 ml in each rat. Spontaneous respiration was stopped by pancuronium bromide (1 mg/kg, i.v.). The bronchomotor tone was measured by the modified Konzett-Rössler method (Konzett & Rössler, 1940) as described in **Experiment 1-1**. The lung was inflated at a fixed volume of air under a constant pressure (5 cmH_2O), and VO was continuously recorded with a combination of pneumotachograph (TP-602T, Nihon Kohden) and integrator (EI-601G, Nihon Kohden). Increase in V.O. means bronchoconstriction in this method. Systemic blood pressure was monitored with a pressure transducer (DX-300, Nihon Kohden) from a cannula inserted into the left carotid artery. All the above parameters were recorded on a polygraph (RM-6000, Nihon Kohden).

ACh solutions (0.001-0.03 %) were cumulatively inhaled each for 3 min to rats by aerosolizing the solution contained in a specially devised plastic cylindrical chamber (capacity: 25 ml) which was introduced in an ultrasonic nebulizer (TUR-3200, Nihon Kohden). The ultrasonic nebulizer with the plastic chamber was placed into the respiratory circuit for the aerosolized mist being inhaled into the airways at each time of ventilation.

The effect of inhaled neurokinin A (NKA) or substance P (SP) on the airway responsiveness to ACh was investigated by using normal rats. Aerosolized NKA (0.001 %), SP (0.01 %) or its vehicle (saline) was inhaled through a tracheal cannula under anesthesia for 3 min. The neutral endopeptidase (NEP) inhibitor, phosphoramidon (dissolved in saline, 5 min before) or thiorphan (dissolved in 10 % dimethyl sulfoxide in saline, 15 min before), was intravenously administered (each 1 or 3 mg/kg) before the inhalation of NKA or SP. Control

rats received respective vehicle injection (0.1 ml/100 g). Immediately after the inhalation of the subbronchoconstrictive concentration of these peptides, the airway responsiveness to ACh was determined as described above. In another series of experiments, the NK-receptor antagonist, [D-Pro^2,D-Trp^{7,9}]-substance P (0.3 or 3.0 nmol/min; 0.1 ml/min of flow), was infused through the right jugular vein under anesthesia from 2 min before phosphoramidon injection. Control animals received saline infusion instead of the antagonist.

Systemic capsaicin pretreatment. The capsaicin treatment was performed as described in **Experiment 4-1**. Animals were treated with a total dose of 125 mg/kg capsaicin, given subcutaneously 10 days before the measurement of airway responsiveness. The capsaicin was given as follows: Day 1, 25 mg/kg; Days 2 and 3, 50 mg/kg of capsaicin. Capsaicin was dissolved in 10 % ethanol-10 % Tween 80-80 % saline. To counteract respiratory impairment caused by capsaicin, the rats were pretreated terbutaline (1 mg/kg, s.c., 60 min before) and aminophylline (25 mg/kg, i.p., 30 min before) before each capsaicin treatment under anesthetization with ketamine (50 mg/kg, s.c.) and thiopental (40 mg/kg, i.p.). The same dosage regimen was used in control animals to deliver an equivalent volume of vehicle (10 % ethanol and 10 % Tween 80 in saline).

Bilateral cervical vagotomy. In some experiments, animals received surgical vagotomy 10 min before experimental procedure. Briefly, rats were anesthetized and ventilated artificially as described above, and the right and left cervical vagi were carefully exposed. The nerve trunks were dissected away from surrounding blood vessels and connective tissues, and were ligated with silk threads about 2-3 mm apart at 15 mm distal to the respective nodose ganglia. Then the nerve trunks were cut between the ligatures. Control rats were subjected to sham operation with the exposure of both vagi alone.

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); capsaicin (Wako Pure Chemical Industries, Osaka, Japan); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane, phosphoramidon, thiorphan, aminophylline, atropine sulfate, carbamylcholine chloride (carbachol) (Sigma, St. Louis, USA); neurokinin A, substance P, [D-Pro^2,D-Trp^{7,9}]substance P (Peptide Institute, Inc., Osaka, Japan).

Capsaicin was dissolved in 10 % ethanol-10 % Tween 80 in saline. Thiorphan was dissolved in 10 % dimethyl sulfoxide in saline. The other drugs used were dissolved and/or diluted in saline.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA).

Results

Inhalation of NKA (0.001 %) or SP (0.01 %) alone to anesthetized normal rats had no significant effect, but NKA slightly augmented the airway responsiveness to inhaled ACh (Figs. 5-1 and 5-2). However, a marked AHR was caused after pretreatment with NKA or SP in the presence of NEP inhibitor, phosphoramidon or thiorphan (1 and 3 mg/kg, *i.v.*), in a dose-dependent manner. The concentration-response curves to inhaled ACh were significantly shifted to the left after treatments with the high dose (3 mg/kg) of phosphoramidon and thiorphan followed by inhalation of NKA ($F_{[1,45]}$=167.2: $p<0.01$ and $F_{[1,50]}$=135.5: $p<0.01$, respectively) (Figs. 5-1 and 5-2) and SP ($F_{[1,45]}$=49.4: $p<0.01$ and $F_{[1,40]}$=75.0: $p<0.01$, respectively) (Figs. 5-3 and 5-4). Pretreatment with either NEP inhibitor (phosphoramidon, thiorphan) alone also induced a marked and significant increase in airway responsiveness to inhaled ACh, in a dose-dependent manner (Figs. 5-5 and 5-6). Figures 5-7 and 5-8 show the % increase in V.O. at respective ACh concentrations, based on the above results. The combination of either NEP inhibitor and NKA had the most potent effect on ACh responses especially at 0.001 to 0.003 %, while the combination of either inhibitor and SP had a weaker and no significant effect as compared with the combination of inhibitors and saline (vehicle for SP) (Figs. 5-7 and 5-8).

The phosphoramidon-induced AHR to inhaled ACh was completely abolished by the systemic capsaicin pretreatment (Fig. 5-9). The concentration-response curve to ACh in normal rats that were treated with the combination of phosphoramidon and NKA (but not SP; data not shown) after systemic capsaicin pretreatment came to the similar level to that of the phosphoramidon-induced increased ACh responsiveness. The intravenous infusion of the non-selective NK-receptor antagonist, [D-Pro^2,D-Trp^{7,9}]SP, also inhibited the phosphoramidon-induced AHR, dose-dependently (Fig. 5-10). These results suggest that endogenous neuropeptides are involved in the AHR induced by NEP inhibitors. Figure 5-11 shows the effect of bilateral cervical vagotomy on the increased airway responsiveness to inhaled ACh after phosphoramidon treatment (3 mg/kg, *i.v.*) in normal rats. The phosphoramidon-induced AHR was partially but significantly attenuated after treatment with vagotomy ($F_{[1,85]}$=6.8: $p<0.05$). In these experiments, the treatment with systemic capsaicin, the high dose of [D-Pro^2,D-Trp^{7,9}]SP or bilateral cervical vagotomy alone did not significantly affect the ACh

response as compared with respective control group.

The bronchoconstriction induced by inhaled carbachol (CCh; unmetabolized by acetylcholinesterase) was significantly greater than that of ACh aerosol at the same concentrations in normal rats (Fig. 5-12). Nevertheless, the bronchoconstriction induced by CCh aerosol was also significantly augmented after *i.v.* treatment with phosphoramidon (Fig. 5-12).

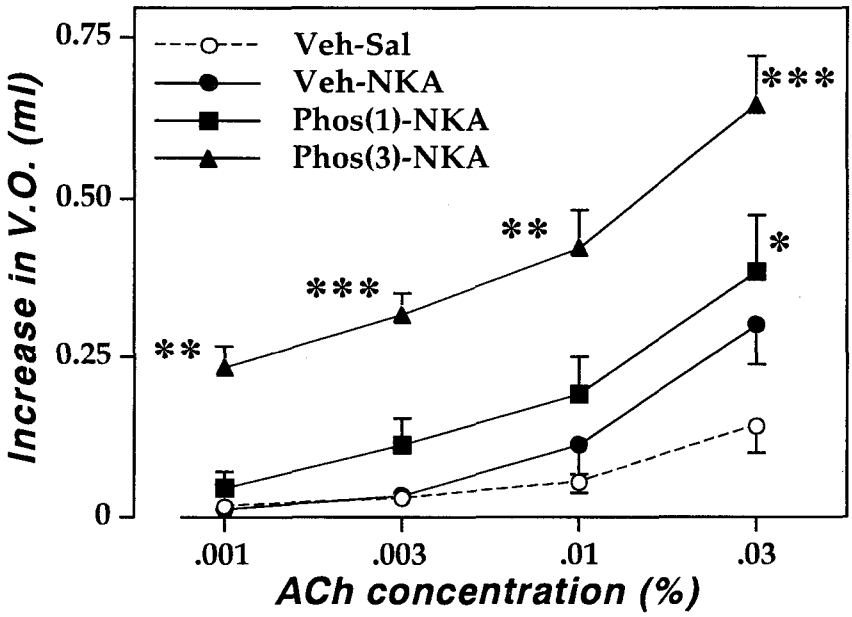


Figure 5-1

The effects of inhaled neurokinin A (NKA) on the airway responsiveness to ACh in normal rats. Phosphoramidon (Phos; 1 or 3 mg/kg, *i.v.*) was dissolved in saline and pretreated 5 min before NKA inhalation under anesthesia. NKA (0.001 %) was dissolved in saline (Sal) and inhaled immediately before measurement of airway responsiveness to ACh. Values are means ± S.E. from 5 to 7 rats. *p<0.05, **p<0.01 and ***p<0.001 vs. Veh-Sal group.

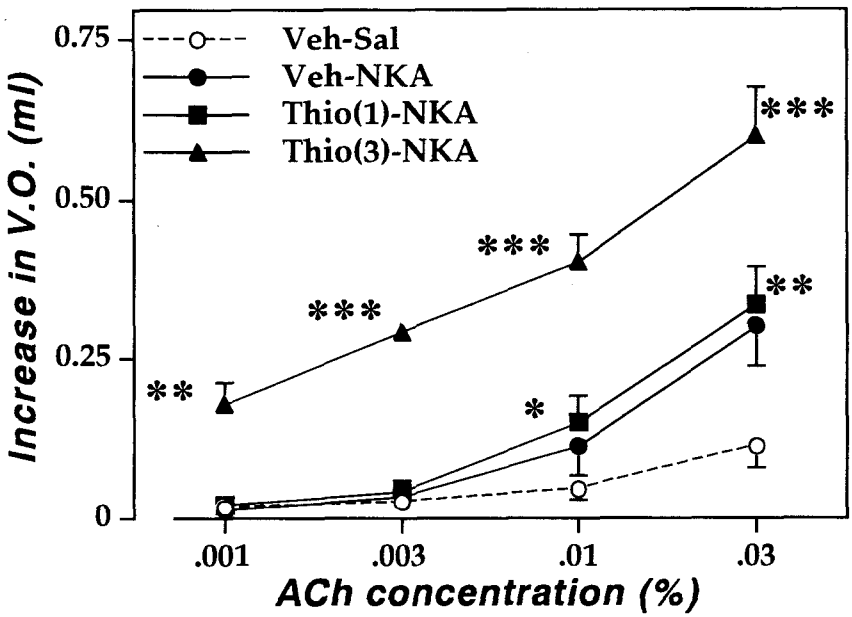


Figure 5-2

The effects of inhaled neurokinin A (NKA) on the airway responsiveness to ACh in normal rats. Thiorphan (Thio; 1 or 3 mg/kg, *i.v.*) was dissolved in 10 % dimethyl sulfoxide in saline and pretreated 15 min before NKA inhalation. NKA (0.001 %) was dissolved in saline (Sal) and inhaled immediately before measurement of airway responsiveness to ACh. Values are means ± S.E. from 5 to 7 rats. *p<0.05, **p<0.01 and ***p<0.001 vs. Veh-Sal group.

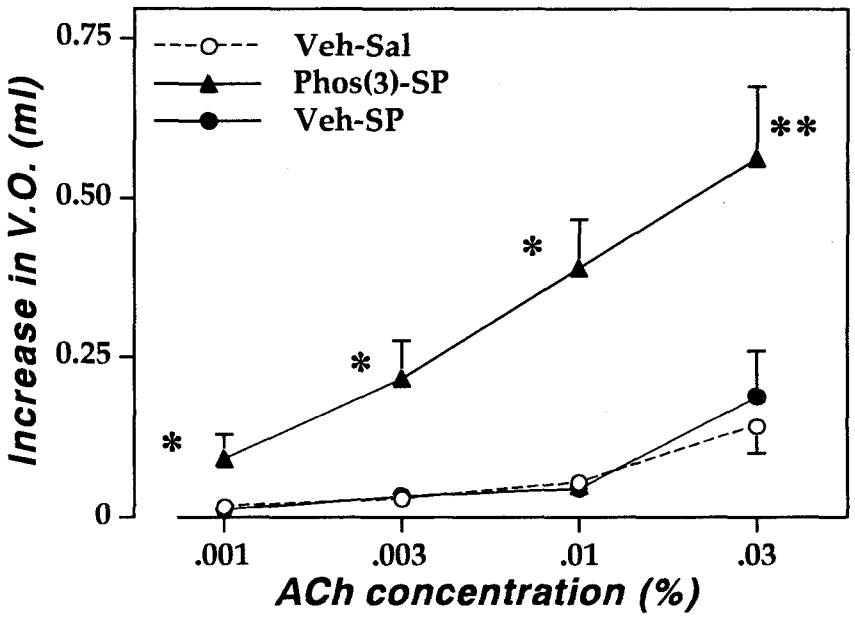


Figure 5-3

The effects of inhaled substance P (SP) on the airway responsiveness to ACh in normal rats. Phosphoramidon (Phos; 1 or 3 mg/kg, *i.v.*) was dissolved in saline (Veh in *a* and *c*) and pretreated 5 min before SP inhalation under anesthesia. SP (0.01 %) was dissolved in saline (Sal) and inhaled immediately before measurement of airway responsiveness to ACh. Values are means ± S.E. from 5 to 7 rats. *p<0.05 and **p<0.01 vs. Veh-Sal group.

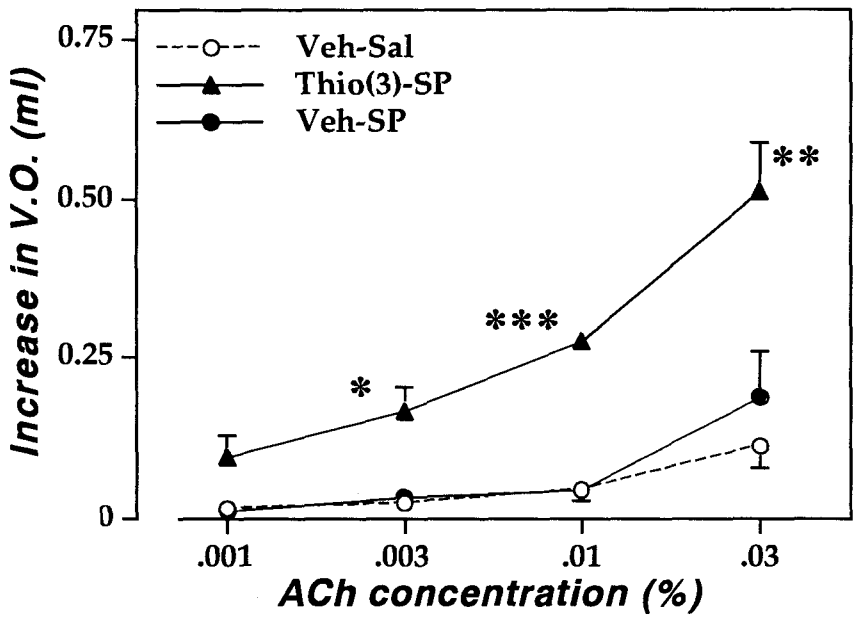


Figure 5-4

The effects of inhaled substance P (SP) on the airway responsiveness to ACh in normal rats. Thiorphan (Thio; 1 or 3 mg/kg, *i.v.*) was dissolved in 10 % dimethyl sulfoxide in saline and pretreated 15 min before SP inhalation. SP (0.01 %) was dissolved in saline (Sal) and inhaled immediately before measurement of airway responsiveness to ACh. Values are means ± S.E. from 5 to 7 rats. *p<0.05, **p<0.01 and ***p<0.001 vs. Veh-Sal group.

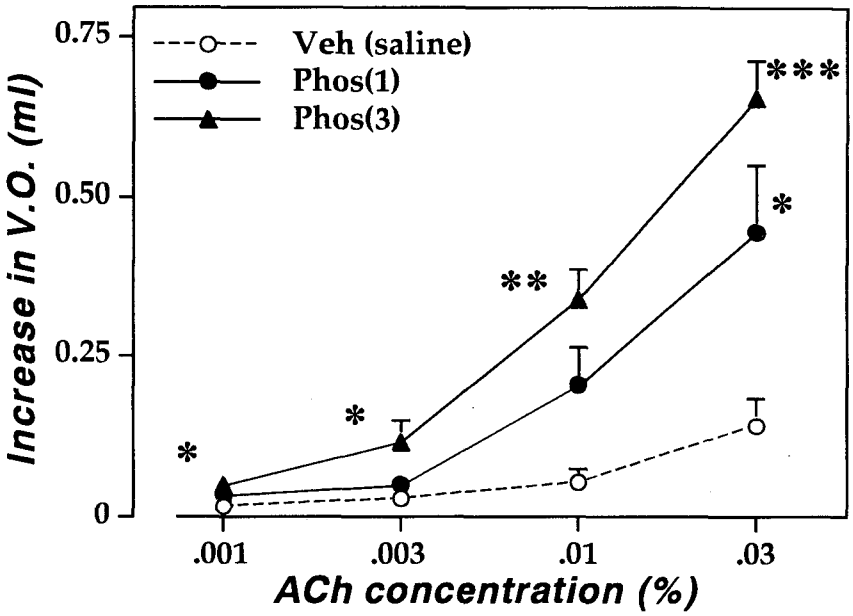


Figure 5-5

The effects of neutral endopeptidase inhibitor, phosphoramidon (Phos), on the airway responsiveness to ACh in normal rats. Phos (1 and 3 mg/kg, *i.v.*) was dissolved in saline and pretreated 5 min before saline (vehicle for NKA and SP) inhalation under anesthesia. Values are means ± S.E. from 5 to 7 rats. *p<0.05, **p<0.01 and ***p<0.001 vs. respective Veh group.

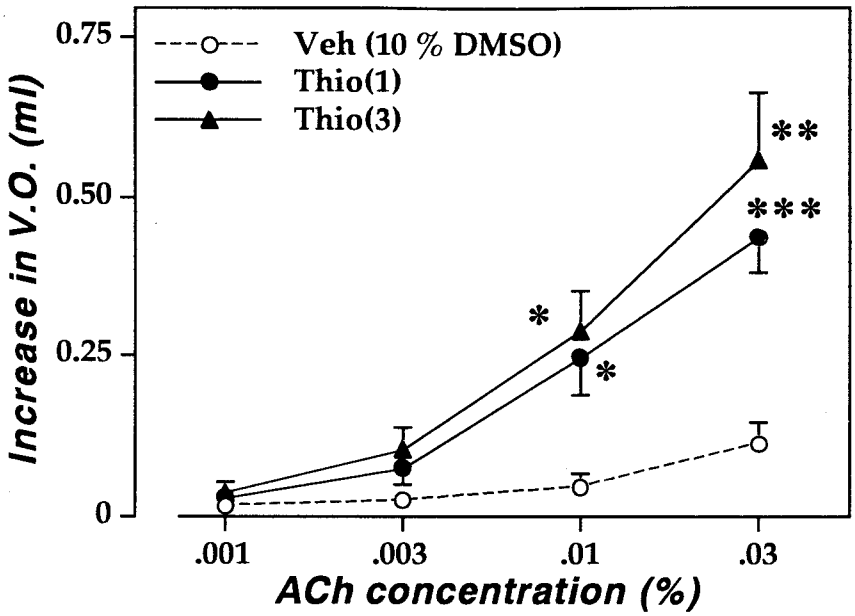


Figure 5-6

The effects of neutral endopeptidase inhibitor, thiorphan (Thio), on the airway responsiveness to ACh in normal rats. Thio (1 and 3 mg/kg, *i.v.*) was dissolved in 10 % dimethyl sulfoxide (DMSO) in saline and pretreated 15 min before saline (vehicle for NKA and SP) inhalation under anesthesia. Values are means ± S.E. from 5 to 7 rats. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. respective Veh group.

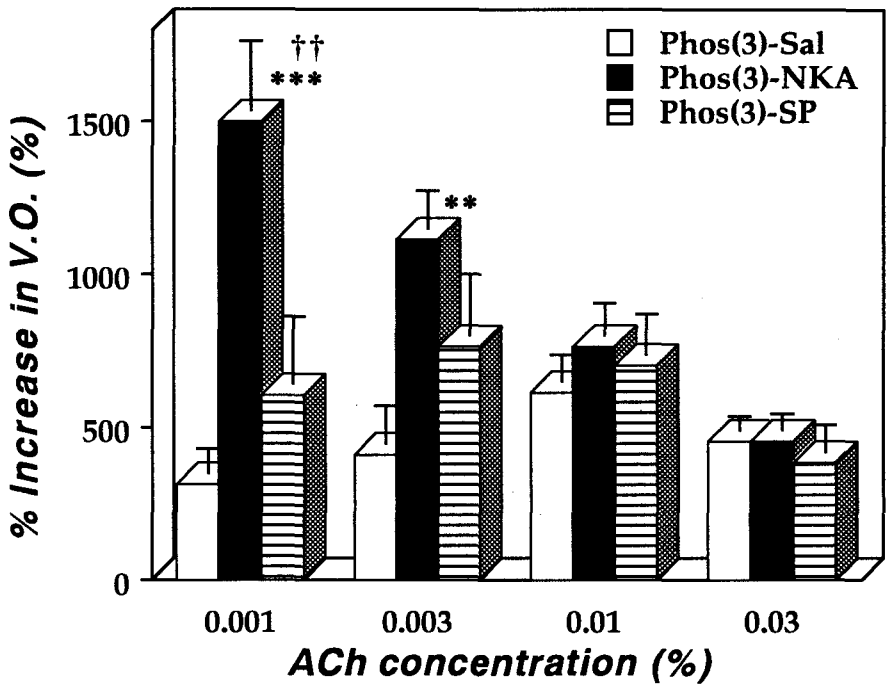


Figure 5-7

Percent changes in ventilation overflow (V.O.) at respective concentration of ACh (0.001-0.03 %) calculated as percentages of respective means of control response (shown in **FIGs. 2** and **3**). Aerosolized NKA (0.001 %; *solid column*), SP (0.01 %; *striped column*) and saline (Sal: vehicle for NKA and SP; *open column*) were inhaled for 3 min immediately before measurement of ACh responsiveness. Phosphoramidon (Phos: 3 mg/kg, *i.v.*; *left panel*) was pretreated 5 min before these peptides inhalation. Values are means ± S.E. from 5 to 7 animals. **p<0.01 and ***p<0.001 vs. Phos(3)-Sal group. ††p<0.01 vs. Phos(3)-SP group.

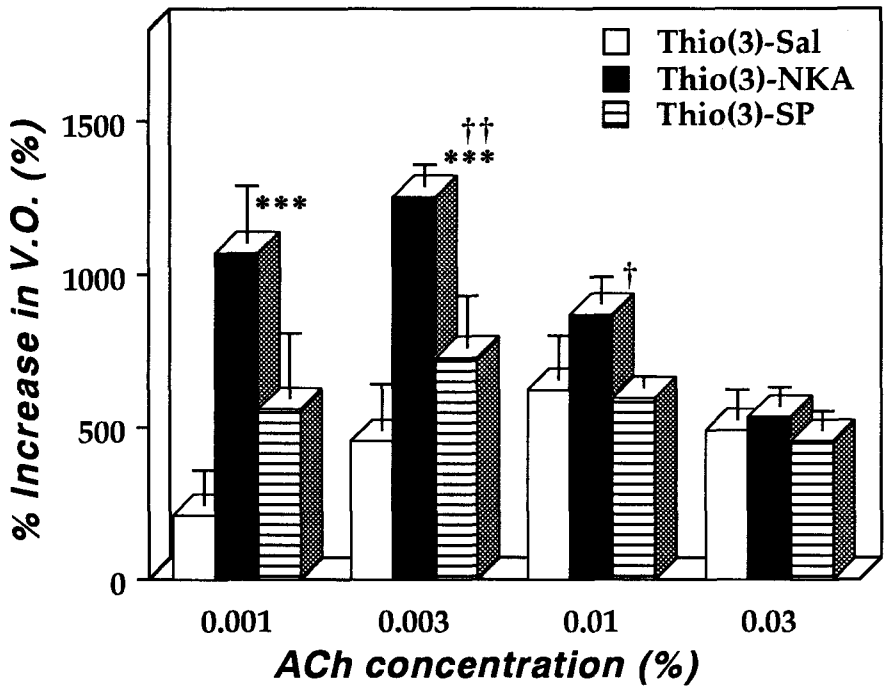


Figure 5-8

Percent changes in ventilation overflow (V.O.) at respective concentration of ACh (0.001-0.03 %) calculated as percentages of respective means of control response (shown in **FIGs. 2** and **3**). Aerosolized NKA (0.001 %; *solid column*), SP (0.01 %; *striped column*) and saline (Sal: vehicle for NKA and SP; *open column*) were inhaled for 3 min immediately before measurement of ACh responsiveness. Thiorphan (Thio: 3 mg/kg, *i.v.*) was pretreated 15 min before these peptides inhalation. Values are means ± S.E. from 5 to 7 animals. ***$p<0.001$ vs. Phos(3)-Sal group. $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ vs. Phos(3)-SP group.

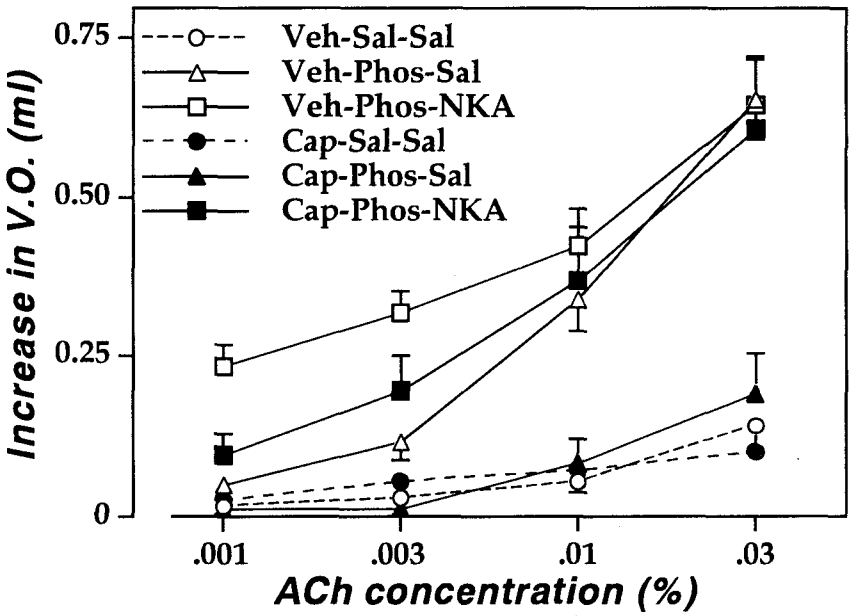


Figure 5-9

The effect of systemic capsaicin pretreatment on the phosphoramidon (Phos)-induced airway hyperresponsiveness to ACh. Some animals were pretreated with capsaicin (Cap) for 3 days (day 1; 25 mg/kg, days 2 and 3; 50 mg/kg, *s.c.*), and 10 days later the examination was made. The other animals were pretreated with vehicle (Veh; 10 % Tween 80 - 10 % ethanol in saline) instead of Cap. At the experimental day (day 11), animals were pretreated with Phos (3 mg/kg, *i.v.*) or saline (Sal) 5 min before inhalation of Sal (vehicle for NKA) under anesthesia. The airway responsiveness to ACh was measured immediately after the inhalation of saline or NKA (0.001 %). Values are means ± S.E. from 4 to 7 animals.

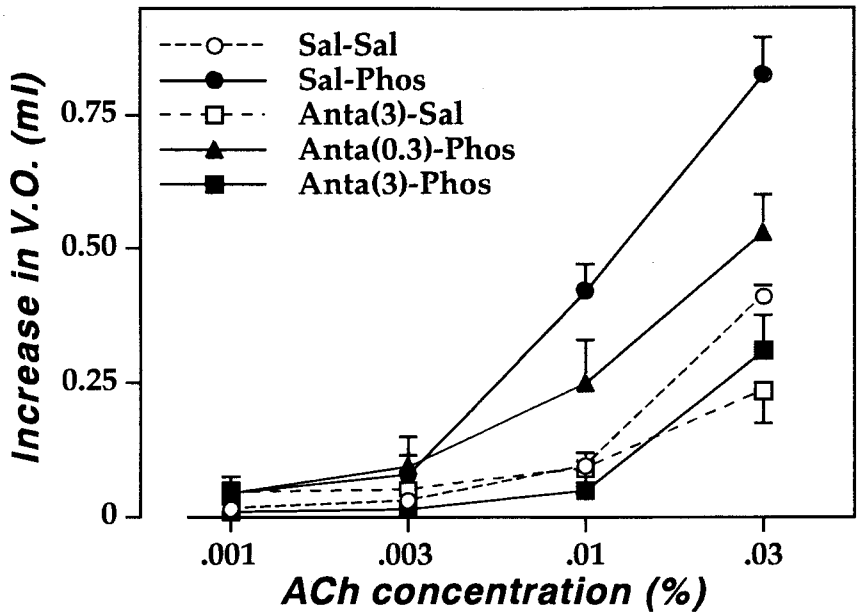


Figure 5-10

The effect of the nonselective NK-receptor antagonist, [D-Pro^2,D-Trp^{7,9}]-SP, on the Phos-induced airway hyperresponsiveness to ACh. The NK-receptor antagonist (0.3 and 3 nmol/min, 0.1 ml/min, respectively) was dissolved in saline and infused from the right jugular vein cannula during the experiment from 2 min before *i.v.* Phos administration. Values are means ± S.E. from 4 to 8 animals.

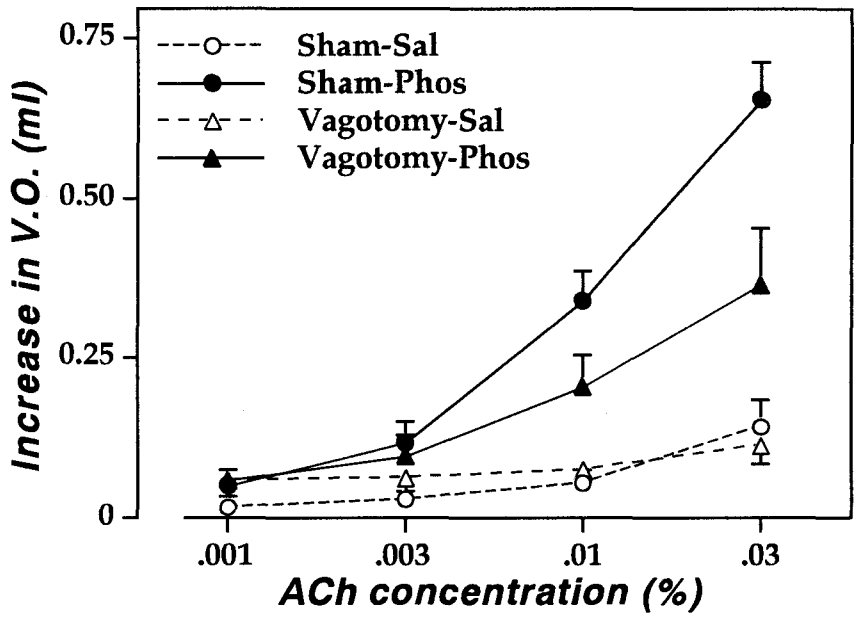


Figure 5-11

The effect of bilateral cervical vagotomy on the Phos-induced airway hyperresponsiveness to ACh. Rats were subjected to surgical vagotomy or sham operation (*see* **METHODS**) 10 min before *i.v.* administration of Phos (3 mg/kg) under anesthesia. Values are means ± S.E. from 4 to 12 animals.

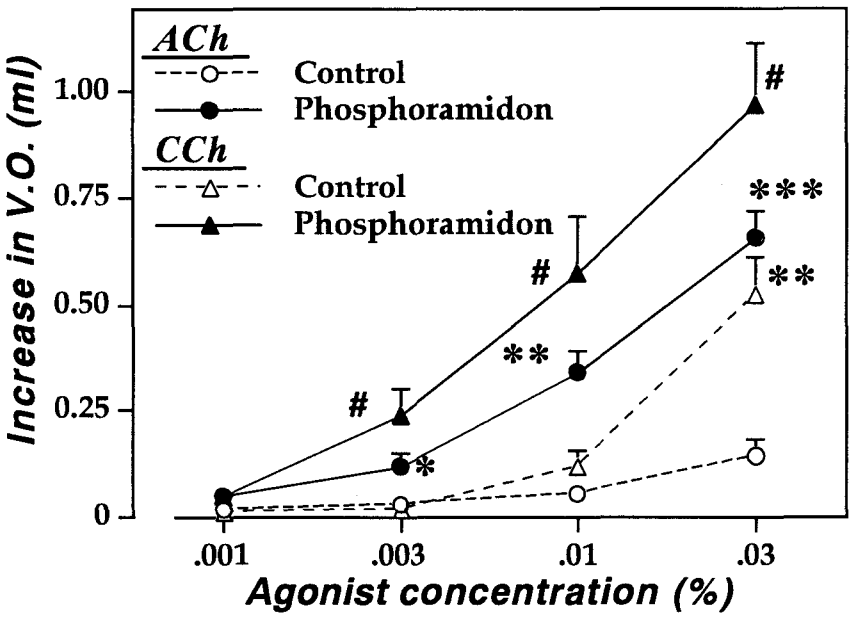


Figure 5-12

Effects of phosphoramidon (3 mg/kg, *i.v.*) on the airway responsiveness to inhaled acetylcholine (ACh) and carbachol (CCh) in normal rats. Phosphoramidon was pretreated 5 min before measurement of airway responsiveness to ACh or CCh under anesthesia. Values are means ± S.E. from 6 to 14 animals. *p<0.05, **p<0.01 and ***p<0.001 vs. ACh control group. #p<0.05 vs. CCh control group.

Experiment 5-2: Effects of exogenous neuropeptides and neutral endopeptidase inhibitors on *in vitro* airway responsiveness to inhaled acetylcholine.

Materials and methods

Animals

Male Wistar rats (8 weeks of age, specific-pathogen-free, 230-310 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water.

***In vitro** study*

Animals were sacrificed by the blow to the head and exsanguinated, and the trachea and bronchus were immediately removed and carefully cleaned of adhering connective tissues. About 5 mm length of the left main bronchus was isolated (8-9 cartilages) and the resultant tissue ring preparation was then suspended in a 10 ml organ bath at a resting tension of 0.5 g. The isometrical contraction of the circular muscle was measured with a force-displacement transducer (TB-612T, Nihon Kohden) and recorded on a polygraph (RM-85, Nihon Kohden). The organ bath contained modified Krebs-Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. The buffer solution was maintained at 37 ℃ and oxygenated with 95 % O_2-5 % CO_2. During an equilibration period in the organ bath, the tissues were washed three to four times at 15 to 20 min intervals and equilibrated slowly to a base line tension of 0.5 g. Fifteen min after the last washing, the concentration-response curve to ACh (10^{-7}-10^{-3} M) was constructed cumulatively. A higher concentration of ACh was successively added after attainment of a plateau response to the previous concentration. After determination of responsiveness to each ACh concentration, the tissues were frequently washed out at 15 to 20 min intervals for following experiments. Under these conditions, reproducible concentration-response curves were obtained on the same tissue strip. In some experiments, NKA (10^{-6} M) or SP (10^{-6} M) was added in the organ bath 10 min before the addition of the lowest concentration of ACh. The combination of enzyme inhibitors, phosphoramidon (10^{-6} M), captopril (10^{-5} M; an inhibitor of angiotensin-converting enzyme) and puromycin (10^{-5} M; an inhibitor of aminopeptidase), was treated 5 min before the addition of these peptides. Some tissues received the pretreatment with tetrodotoxin (TTX; 10^{-6} M) in the organ bath 5 min prior to the combination of enzyme inhibitors.

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); capsaicin, Tris (Wako Pure Chemical Industries, Osaka, Japan); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane, phosphoramidon, thiorphan, aminophylline, atropine sulfate, tetrodotoxin, carbamylcholine chloride (carbachol) (Sigma, St. Louis, USA); neurokinin A, substance P, [D-Pro^2,D-Trp^{7,9}]substance P (Peptide Institute, Inc., Osaka, Japan); Evans blue (Merck Co., New Jersey, USA) and [^{3}H]QNB (l-[benzilic-4,4'-^{3}H]-quinuclidinyl benzilate, New England Nuclear, Stevenage, UK).

Capsaicin was dissolved in 10 % ethanol-10 % Tween 80 in saline. Thiorphan was dissolved in 10 % dimethyl sulfoxide in saline. The other drugs used in the *in vivo* and *in vitro* studies were dissolved and/or diluted in saline and modified Krebs-Henseleit solution, respectively. The drugs used for the binding assay was dissolved and diluted in Tris HCl buffer (pH 7.4) except for [^{3}H]QNB (in water).

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data, Cochran-Cox test or two way analysis of variance (ANOVA).

Results

In the presence of phosphoramidon (10^{-6} M) plus NKA (10^{-6} M), the concentration-response curve to ACh was significantly shifted to the left ($F_{[1,198]}$=21.0: $p<0.01$), although not so potently as compared with the case of *in vivo* study. On the other hand, phosphoramidon alone had no effect in this case (data not shown). The combination of phosphoramidon and SP (10^{-6} M) had no effect on the ACh response. The increased bronchial responsiveness to ACh after treatment with phosphoramidon plus NKA was significantly inhibited by pretreatment with tetrodotoxin (TTX; 10^{-6} M, $F_{[1,198]}$=10.6: $p<0.01$); the ACh concentration-response curve after pretreatment with TTX was significantly shifted lower than that after pretreatment with three respective vehicles ($F_{[1,198]}$=9.4: $p<0.01$), although the maximal responses to 10^{-3} M ACh had no significant difference.

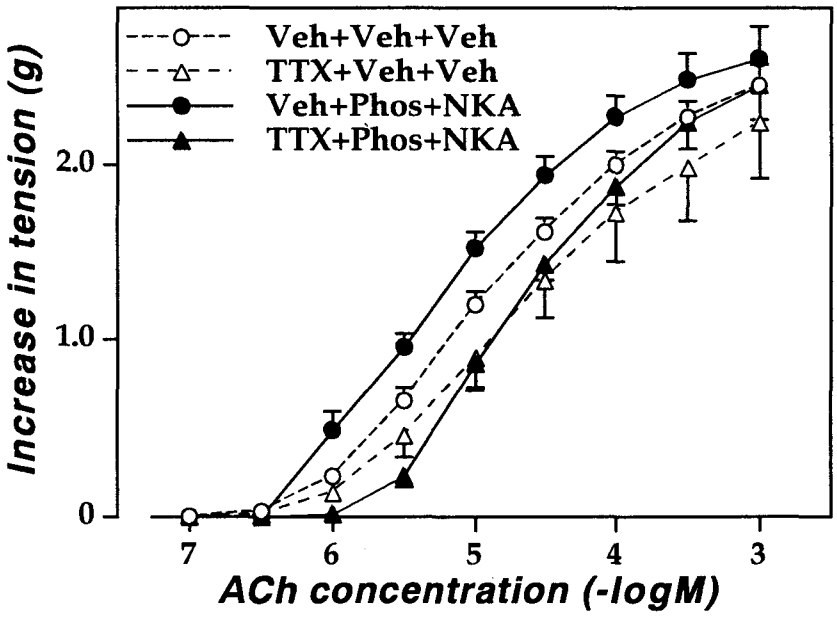


Figure 5-13

The effects of NKA (10^{-6} M) on the responsiveness to ACh of the isolated main bronchial tissue of normal rats. NKA (10^{-6} M) was added in the organ bath 10 min before administration of the lowest concentration of ACh, and phosphoramidon (Phos; 10^{-6} M) was treated 5 min before the addition of NKA. Captopril and puromycin (10^{-5} M) were present in the organ bath in all experiments. Values are means ± S.E. from 7 to 16 animals. Tetrodotoxin (TTX; 10^{-6} M) was pretreated 5 min prior to the addition of Phos. In *in vitro* study, Phos alone had no significant effect on the ACh responsiveness (data not shown). Statistical significance (by **ANOVA**) was observed between the groups of Veh+Veh+Veh vs. TTX+Veh+Veh ($F_{[1,198]}$=9.4: $p<0.01$), Veh+Phos+NKA ($F_{[1,198]}$=21.0: $p<0.01$) and TTX+Phos+NKA ($F_{[1,198]}$=10.6: $p<0.01$), respectively, but no significant difference was observed in TTX+Veh+Veh vs. TTX+Phos+NKA ($F_{[1,126]}$=0.23: $p>0.05$).

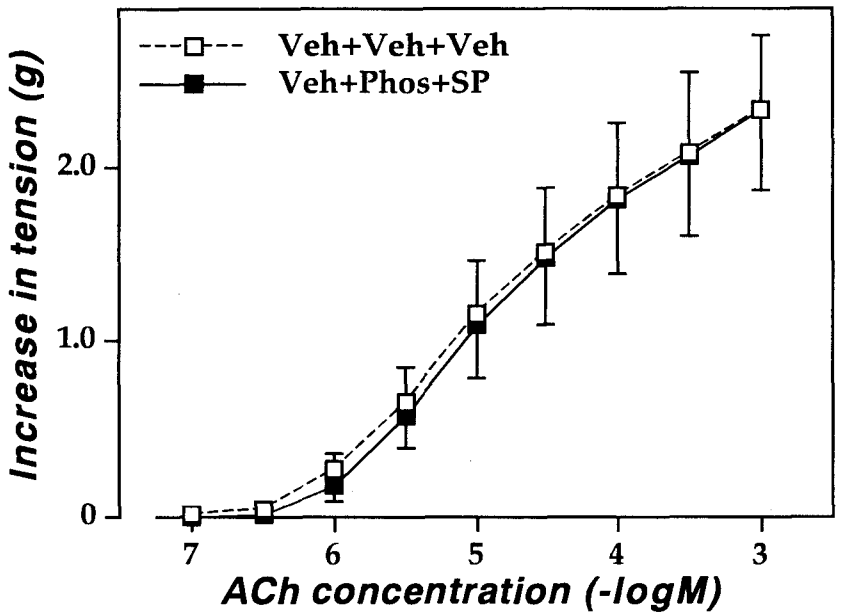


Figure 5-14

The effects of SP (10^{-6} M) on the responsiveness to ACh of the isolated main bronchial tissue of normal rats. SP (10^{-6} M) was added in the organ bath 10 min before administration of the lowest concentration of ACh, and phosphoramidon (Phos; 10^{-6} M) was treated 5 min before the addition of SP. Captopril and puromycin (10^{-5} M) were present in the organ bath in all experiments. Values are means ± S.E. from 7 to 8 animals. In *in vitro* study, Phos alone had no significant effect on the ACh responsiveness (data not shown).

Experiment 5-3: Effects of neurokinin A and phosphoramidon on airway microvascular leakage.

Materials and methods

Animals

Male Wistar rats (8 weeks of age, specific-pathogen-free, 230-310 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water.

Determination of airway microvascular leakage

Airway microvascular leakage was quantified by the method described in **Experiment 1-6**. Rats were anesthetized and ventilated as described above, and Evans blue dye (E.B.; 30 mg/ml in saline, filtered with paper filter), 30 mg/kg, and heparin, 600 U/kg, were administered *i.v.* 3 min after phosphoramidon administration (3 mg/kg, *i.v.*). Two minutes after the dye injection, NKA (0.001 %) and then increasing concentrations of ACh (0.001-0.03 %) were inhaled by the method described above. Control rats received saline injection and saline inhalation instead of phosphoramidon and NKA, respectively. Twenty minutes after the E.B. administration, the animals were exsanguinated from the abdominal aorta, and then the thorax was opened and two blunt-ended 13-gauge needles were inserted into the left and right ventricles toward the aorta and pulmonary artery, respectively. The right atrium and pulmonary veins were incised to allow the outflow of perfusate, and the airways and lungs were perfused with 40-50 ml of 1 % paraformaldehyde in citrate-buffered saline (pH 3.5) to remove intravascular dye and to fix the tissues. After then, the trachea, main bronchus and lungs were removed and their wet weights were measured.

E.B. was extracted by incubating the tissues in 1 ml (trachea and main bronchus) or 3 ml (lungs) of 100 % formamide at 55 °C for 24 hr, and its concentration was determined by light absorbance at 620 nm (U-2000 spectrophotometer, Hitachi) and by interpolation on a standard curve of E.B. concentrations (3.125-50 μg/ml). E.B. content from each sample was expressed as nanogram per milligram wet weight of tissue.

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo); pancuronium bromide (Sankyo Co., Tokyo); urethane, phosphoramidon (Sigma, St. Louis, USA);

neurokinin A (Peptide Institute, Inc., Osaka, Japan) and Evans blue (Merck Co., New Jersey, USA).

The drugs used in this study were dissolved and/or diluted in saline.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

Results

The microvascular leakage in the trachea, main bronchus and lungs was not significantly changed by the treatment with phosphoramidon and NKA (Table 5-1).

Table 5-1. Effect of the combination of phosphoramidon (Phos) and neurokinin A (NKA) on the Evans blue dye (E.B.) exudation of the trachea, main bronchus and lungs of anesthetized, normal rats

Treatment	E.B. exudation (ng/mg wet weirht)		
	Trachea	Bronchus	Lungs
Sal-Sal (n=5)	20.7±2.3	18.1±2.6	13.7±1.7
Phos-NKA (n=5)	21.8±4.4^{ns}	19.0±1.6^{ns}	15.8±1.9^{ns}

The E.B. exudations of the trachea, main bronchus and lungs are expressed as nanogram of extravasated E.B. per milligram of respective tissue wet weight (mean ± S.E.). Control group (Sal-Sal) received intravenous administration of saline (Sal) and saline inhalation instead of the combination of phosphoramidon (3 mg/kg) and NKA (0.001 %), respectively, before inhalation of cumulative concentrations of ACh (0.001-0.03 %). *ns*; no significant difference vs. Sal-Sal group.

Experiment 5-4: Effects of neuropeptides on the muscarinic receptor property in airways.

Materials and methods

Animals

Male Wistar rats (8 weeks of age, specific-pathogen-free, 230-310 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water.

Binding assay

Airway tissues and lungs were removed as described in **Experiment 4-3**. The tracheae, main bronchi and superior lobus of left lungs were collected from 7-9 animals to obtain one preparation for one number of experiment. These tissue segments were homogenized with Physcotron (NITI-ON, Co. Ltd., Japan: 5 sec x 6; level max) in 10 vol of ice-cold 0.32 M sucrose. These whole tissue homogenates were centrifuged (1,500 g, 4 °C for 15 min) and then the resultant supernatants were recentrifuged (50,000 g, 4 °C for 15 min). The resultant pellets were resuspended in 10 vol of ice-cold 50 mM tris(hydroxymethyl)aminomethane (Tris) HCl buffer (pH 7.4) and homogenized (5 sec; level max). The suspensions were again centrifuged (50,000 g, 4 °C for 15 min) and the pellets were resuspended in 10 vol of ice-cold 50 mM Tris HCl buffer (pH 7.4). These membrane preparations were stored at -80 °C until use. The protein concentrations of these preparations were determined by the method of Lowry *et al.* (1951) in triplicate with bovine serum albumin as a standard.

First, we performed [^{3}H]QNB binding assay by using the membrane preparations from normal rats to characterize the [^{3}H]QNB binding sites as muscarinic ACh receptors. Briefly, the assay tubes (total volume of 500 μl containing 0.1 mg protein of the membrane preparation) were incubated with different concentrations of [^{3}H]QNB (specific activity 38.8 Ci/mmol, final concentration of 15.625-2,000 pM) at 25 °C for 60 min. At the end of incubation, the suspension was rapidly filtrated through polyethylenimine-coated Whatman GF/C glass fiber filter to separate bound [^{3}H]QNB from free [^{3}H]QNB. The filter was washed 3 times with 5 ml of 50 mM Tris HCl buffer (pH 7.4), and then the radioactivity was counted in 10 ml of scintillation fluid (Aquasol-2; New England Nuclear, Stevenage, UK) by a liquid scintillation counter (LSA-1600CA liquid scintillation analyzer, Packard). Specific binding was calculated by subtracting nonspecific binding in the presence of atropine (10^{-6} M) from

total binding in the absence of atropine. These procedures were performed in duplicate, and then the Scatchard analysis of [^{3}H]QNB binding was done.

By using these membrane preparations from normal rats, the effect of phosphoramidon, NKA or SP on the [^{3}H]QNB binding was determined in triplicate. The assay tube (total volume of 500 μl containing 0.1 mg protein of the membrane preparation) was preincubated with the combination of peptidase inhibitors, 10^{-6} M of phosphoramidon and 10^{-5} M of captopril and puromycin, at 37 °C. Ten min later, NKA or SP (10^{-6} M) was added in the assay tube and preincubated at 37 °C for 10 min. After these preincubation periods, the binding assay was done by using 1 nM of [^{3}H]QNB, as described above.

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); Tris (Wako Pure Chemical Industries, Osaka, Japan); phosphoramidon, atropine sulfate (Sigma, St. Louis, USA); neurokinin A, substance P (Peptide Institute, Inc., Osaka, Japan) and [^{3}H]QNB (l-[benzilic-4,4'-^{3}H]-quinuclidinyl benzilate, New England Nuclear, Stevenage, UK).

The drugs used for the binding assay was dissolved and diluted in Tris HCl buffer (pH 7.4) except for [^{3}H]QNB (in water).

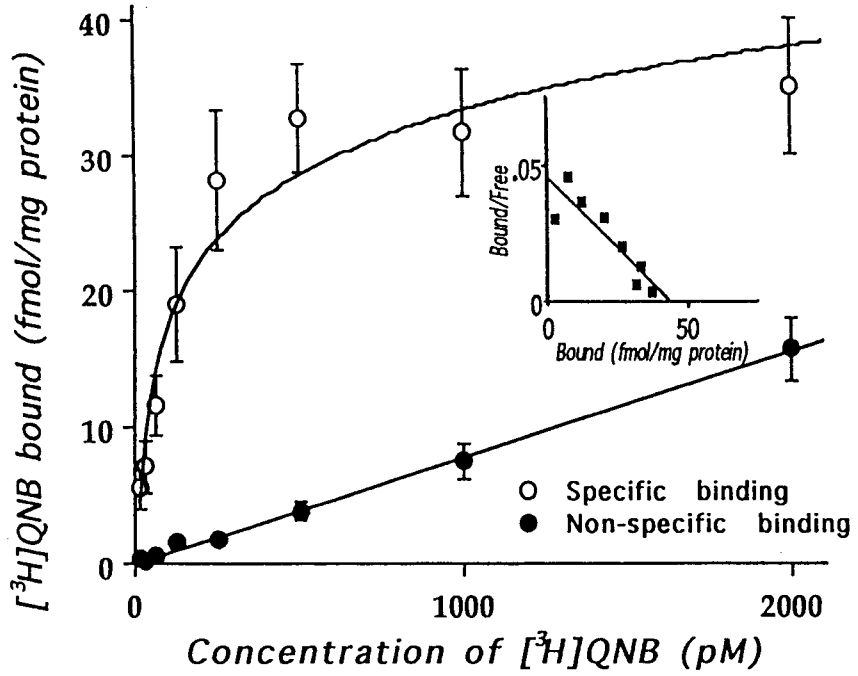
Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Student's *t*-test for unpaired data or Cochran-Cox test.

Results

The upper panel of figure 5-15 shows the saturation curve obtained from binding assay using the crude membrane preparation from normal rat airway tissues. The nonspecific component determined in the presence of 10^{-6} M atropine was linear (r=0.997). Specific binding of [^{3}H]QNB to rat airway tissue membranes was saturable and of high affinity, with a dissociation constant (Kd) of 194.9±44.9 pM and a maximal number of binding sites (Bmax) of 37.7±4.2 fmol/mg protein (n=6).

The lower panel of figure 5-15 shows the effect of NKA or SP with/without phosphoramidon, captopril and puromycin on [^{3}H]QNB binding sites of the above membrane preparations. These peptides and/or inhibitors had no effect on [^{3}H]QNB binding sites at the [^{3}H]QNB concentration of 1 nM which covers almost all the specific binding sites of these preparations (Fig. 5-15, upper panel).


40
30
20
10
0
0
1000
2000
[3H]QNB bound (fmol/mg protein)
Concentration of [3H]QNB (pM)
Specific binding
Non-specific binding
Bound/Free
.05
0
0
50
Bound (fmol/mg protein)

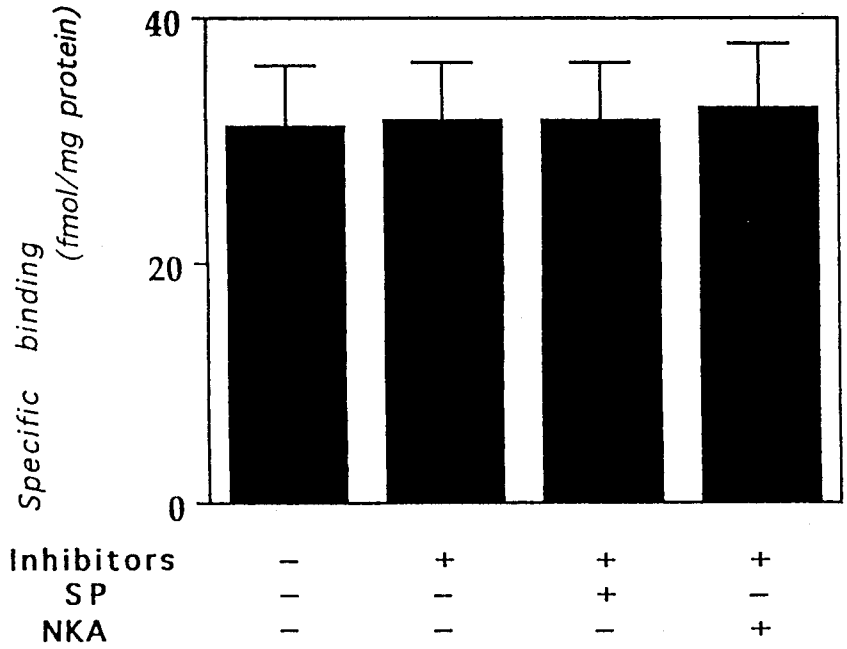

40
20
0
Specific binding (fmol/mg protein)
Inhibitors – + + +
SP – – + –
NKA – – – +

Figure 5-15

(Legend for **Figure 5-15**)

Upper: The saturation curve for [^{3}H]QNB binding to the airway tissue crude membrane preparations from normal rats. The membrane preparations were incubated in duplicate with increasing concentrations of [^{3}H]QNB (15.625-2000 pM) in the absence or presence of atropine (10^{-6} M) for 60 min at 25 °C. Specific binding (*open circle*) was obtained by the difference between the total [^{3}H]QNB binding and nonspecific binding (*closed circle*) as defined in the presence of atropine. Values are means ± S.E. from 6 experiments. *Inset*: Typical Scatchard plots of data from above preparations. The Kd and Bmax values are 194.9±44.9 pM and 37.7±4.2 fmol/mg protein, respectively, from 6 experiments. *Lower*: The effects of NKA and SP with/without phosphoramidon (each 10^{-6} M) on [^{3}H]QNB binding sites of the membrane preparations of normal rat airways. The membrane preparations were preincubated with NKA or SP with/without inhibitors (phosphoramidon, 10^{-5} M of captopril and puromycin) for 10 min, and then [^{3}H]QNB binding assay was performed in triplicate. Values are means ± S.E. from 6 experiments.

Discussion

In the present study, exogenously administered neuropeptides (especially NKA) in the presence of NEP inhibitors caused AHR to ACh in normal nonsensitized animals in both *in vivo* and *in vitro* experiments. Interestingly, in our present study, intravenous administration of NEP inhibitor, phosphoramidon or thiorphan (1 and 3 mg/kg) alone, also markedly increased airway responsiveness to ACh, dose-dependently. This finding strongly suggests a hypothesis that endogenously released sensory neuropeptides might cause the increased airway responsiveness. On the other hand, exogenously administered NKA itself, at the concentration used, had no contractile and vascular permeable effects even in the presence of NEP inhibitors in normal rats.

Sekizawa *et al.* (1987b) reported that pretreatment with NEP inhibitor (Leu-thiorphan) of the isolated ferret trachea potentiated the SP and electrical field stimulation (EFS) responsiveness. This event might be explicable by the notion that the NEP existed on epithelial and smooth muscle membranes cleaves exogenously applied and endogenously released neuropeptides such as SP and NKA. So, if the NEP activity is blocked by its inhibitor, the contractile response to these neuropeptides may be augmented. Similar results were obtained in *in vivo* studies using guinea pigs (Lötvall *et al.*, 1990b; Thompson & Sheppard, 1988). However, in our study, the airway responsiveness to inhaled ACh (not neuropeptides) was significantly augmented after treatment with NEP inhibitors alone in rats. This observation was an unexpected finding for us because Thompson and Sheppard (1988) reported that pretreatment with phosphoramidon (0.5 mg/animal, *i.v.*) had no effect on the airway responsiveness to inhaled ACh in guinea pigs. In our results, not only phosphoramidon but also thiorphan caused ACh hyperresponsiveness *in vivo*, indicating that inhibition of NEP activity might be involved in this hyperresponsiveness. Furthermre, this AHR induced by phosphoramidon was also observed in case of using CCh (unaffected by acetylcholinesterase) aerosol as a contractile agonist (Fig. 5-12), indicating that inactivation of acetylcholinesterase might not be involved in this AHR. Although the reason why these results were observed in our study using rats is difficult to explain, species differences (*e.g.* distribution of NEP activity, innervation of neuropeptides-containing nerves) might be implicated in this disagreement.

In the present study, the phosphoramidon-induced AHR to inhaled ACh was completely abolished by systemic capsaicin treatment, and dose-dependently inhibited by a tachykinin receptor antagonist, [D-Pro^2,D-Trp^{7,9}]SP. In the presence of phosphoramidon, the inhalation of NKA, but not SP, to the capsaicinized rats also caused AHR to ACh aerosol. Furthermore, it was reported that a cholinergic agonist had an ability to release neuropeptides such as SP and

NKA in the guinea pig isolated trachea (Martins *et al.*, 1991). This suggests that inhaled ACh even slightly caused the release of sensory neuropeptides, which, especially NKA, were responsible for the NEP inhibitors-induced AHR. However, NKA itself had no direct and/or indirect effects on the bronchial contractile response in Wistar rats, because the exogenous administration of NKA (up to the concentration of 10^{-4} M) to the rat isolated bronchial tissues even in the presence of NEP inhibitors caused no contractile response (data not shown).

Lötvall *et al.* (1990a) have shown that inhalation of SP aerosol to anesthetized guinea pigs caused an increase in lung resistance accompanied by a significant E.B. extravasation and edema in the airways; this airway edema contributed to the airway obstruction. It has been also reported that intravenously administered NKA has an ability of edema formation in guinea pig airways (Tousignant *et al.*, 1993). There are some findings demonstrating a correlation between the airway inflammatory events and nonspecific AHR induced by ozone, cigarette smoke, toluene diisocyanate and/or antigen in many species including human (Chung, 1986). The inhaled NKA concentration used in the present study, however, had no significant effect on the E.B. exudation (Table 5-1), indicating that the increased bronchomotor response to ACh observed in the present study is not due to airway edema. The pretreatment with phosphoramidon plus NKA, even at the subthreshold concentrations for inducing inflammation, induced the AHR to ACh which was not accompanied by increased E.B. exudation (an index of airway inflammation) in normal nonsensitized rats. It is therefore interestingly suggested that the airway inflammation, at least enhanced vascular permeability, might not be an essential condition for inducing the AHR, at least in normal rats.

Endogenous substances including somatostatin are known to affect the binding site of muscarinic ACh receptor in the brain (Miyoshi *et al.*, 1986). Since we suspected that the postjunctional ACh binding sites might be affected by NKA, the effect of NKA on [^{3}H]QNB binding sites was examined. As shown in Fig. 5-15, the tissue membrane preparations obtaind from the normal rat airways had one high affinity binding site of [^{3}H]QNB, and NKA had no significant effect on [^{3}H]QNB binding. This result suggests that the AHR to ACh induced by phosphoramidon plus NKA is not due to the increment of the postjunctional muscarinic ACh receptors. The finding that the *in vitro* ACh hyperresponsiveness induced by exogenously applied phosphoramidon plus NKA was completely abolished by TTX (Fig. 5-13) also supports that postjunctional muscarinic ACh receptors were unaffected by the treatment with phosphoramidon plus NKA.

The phosphoramidon-induced AHR to inhaled ACh was significantly, but partially attenuated by bilateral cervical vagotomy (Fig. 5-11). This finding raises the possibility that the enhanced vagal reflex might be, at least in a part, involved in this AHR. In addition, in our *in*

vitro study, the phosphoramidon plus NKA-induced AHR to ACh was completely abolished by TTX treatment. Furthermore, the contractile response to ACh itself was significantly attenuated by TTX treatment (Fig. 5-13). These observations imply that the exogenously applied ACh acts on not only airway smooth muscles directly but also airway nerve endings and/or ganglions which causes the release of sensory neuropeptides and other contractile transmitter(s); the released neuropeptides, especially NKA, might augment the release of contractile transmitter(s) in the presence of NEP inhibitors. Hall *et al.* (1989) reported that low concentration of tachykinins, that had little effect on intraluminal pressure, caused an increase in the contractions elicited by stimulation of the preganglionic vagal nerve fibers and postganglionic (transmural) stimulation in guinea pig innervated tracheal tube preparations; the order of potency being NKA >> SP. They concluded that tachykinins act on NK_2 receptors on postganglionic pulmonary parasympathetic nerve terminals, and that activation of the neuronal receptors may increase the probability of transmitter release from the nerve terminals. From these findings, it is possible that activation of NK_2 receptors on postganglionic parasympathetic nerve by NKA increases the release of ACh, which in turn augments the bronchoconstriction induced by exogenously applied ACh. Although the reason is not identified why phosphoramidon alone had no effect in *in vitro* study, the network of NKA-containing innervation might be much reduced by surgical operations in the isolated main bronchial preparation; the reduced regions possibly importantly participate in the effects of NKA, in terms of presynaptic neurons, vagal reflex, autonomic ganglia, etc. On the other hand, in our *in vivo* system, the preganglionic vagal nerve also seems to be activated, because the ACh hyperresponsiveness induced by phosphoramidon was partly attenuated by bilateral cervical vagotomy (Fig. 5-11). These complex, enhanced probability of transmitter release might be involved in this AHR *in vivo*.

In summary, we demonstrated that 1) treatment with NEP inhibitor plus NKA of normal nonsensitized rats caused AHR to ACh both *in vivo* and *in vitro* without increased vascular permeability, 2) *i.v.* treatment with NEP inhibitor alone also induced AHR to inhaled ACh in normal nonsensitized rats, and 3) this AHR was completely blocked by capsaicin treatment and inhibited by NK-receptor antagonist, dose-dependently. These findings suggest an important role of endogenous neuropeptides in the pathogenesis of AHR. We also demonstrated that 4) the AHR to ACh induced by *i.v.* administration of phosphoramidon and that induced by *in vitro* treatment with phosphoramidon plus NKA were significantly attenuated by pretreatment with bilateral cervical vagotomy and TTX, respectively, suggesting that sensory neuropeptides, especially NKA, enhance the vagal reflex and/or probability of transmitter release. Activation of sensory nerve by various stimuli causes the release of sensory

neuropeptides and the released neuropeptides, especially NKA, in turn enhance the probability of transmitter release. The decreased NEP activity and enhanced transmitter release might be, at least in a part, involved in the pathogenesis of AHR. In our present study using NKA, SP and a nonselective tachykinin antagonist, although the selective NK_1- and NK_2-receptor antagonists (or agonists) were not used, the NK_2-receptors might be predominantly involved in these enhanced transmitter release because NKA had more potent effect than SP both *in vivo* and *in vitro* studies.

CHAPTER 6:
Alteration of Ca^{2+} mobilization contributing to the bronchial smooth muscle constriction after repeated antigenic challenge

Introduction

Although various results were reported about the *in vitro* responsiveness of airway smooth muscles isolated from asthmatic patients (Vincenc *et al.*, 1983; Roberts *et al.*, 1984; Schellenberg & Foster, 1984; Cerrina *et al.*, 1986; Goldie *et al.*, 1986; De Jongste *et al.*, 1987; Bai, 1990), it has also been reported that asthmatic patients have hyperresponsiveness both *in vivo* and *in vitro* (Roberts *et al.*, 1984; Schellenberg & Foster, 1984; De Jongste *et al.*, 1987; Bai, 1990). For instance, airway smooth muscles obtained from *in vivo* hyperresponsive patients have *in vitro* hyperresponsiveness to methacholine (Roberts *et al.*, 1984). Likewise, we have recently demonstrated both *in vivo* and *in vitro* AHR to acetylcholine (ACh) in rats that were actively sensitized and repeatedly challenged with aerosolized antigen (**CHAPTERS 1** and **2**).

Elucidation of the Ca^{2+} mobilization mechanisms in airway smooth muscles is important not only for the understanding of the regulatory mechanisms of smooth muscle contraction, but also for the better understanding of the mode of action of the drugs that modulate smooth muscle activities. The roles of smooth muscle contractions vary greatly among various organs to which the muscle cells belong, and 'diversities' in the regulation of smooth muscle activities has been often emphasized. Difficulty in the study of the Ca^{2+} mobilization in smooth muscle cells has in this apparent complexity. Ca^{2+} ions required for the activation of smooth muscle contraction may come from the outside of the cells through ion channels on the plasma membrane or from the intracellular storage site(s) via Ca^{2+} release mechanisms located on the organella(s). Hitherto, there has been no report about the study in which Ca^{2+} mobility change in bronchial smooth muscle cells by AHR was examined.

To understand the mechanisms of the increased responsiveness to ACh, we therefore examined the effect of extracellular Ca^{2+} removal on ACh-induced constriction of bronchial rings from airway hyperresponsive rats. We also investigated the Ca^{2+}-induced constriction of bronchial rings from those rats that were depolarized with ACh or high K^+ in Ca^{2+}-free medium. The isolated bronchi from airway hyperresponsive rats were compared with bronchial rings from non-sensitized normal animals.

Experiment 6-1: Effect of repeated antigenic challenge on acetylcholine-induced bronchoconstriction in Ca^{2+}-free medium.

Materials and methods

Sensitization and antigenic challenge

The method for inducing AHR was described in **Experiment 1-2**. Male Wistar rats (6 weeks of age, specific-pathogen-free, 170-190 g) were purchased from Charles River Japan, Inc. and housed under standard laboratory conditions with free access to food and water. Animals were sensitized with 2,4-dinitrophenylated *Ascaris suum* extract (DNP-Asc, 2 mg protein, s.c.) together with *Bordetella pertussis* ($2X10^{10}$) as an adjuvant and were boosted by DNP-Asc (0.5 mg protein, i.m.) 5 days later. Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc (6 mg protein/ml, 5-6 ml) with an ultrasonic nebulizer (TUR-3000, Nihon Kohden, Tokyo) for 20 min in conscious state in a plexiglass box (300X200 mm, height: 150 mm). Then the animals were subjected to 3 times repeated antigenic challenge every 48 h with the same inhalational challenge method described in **Experiment 1-2**.

In vitro determination of airway response

Animals were sacrificed by the blow to the head and exsanguinated, and the trachea and bronchus were immediately removed and carefully cleaned of adhering connective tissues. About 5 mm length of the left main bronchus was isolated (8-9 cartilages) and the resultant tissue ring preparation was then suspended in a 10 ml organ bath by two stainless-steel wires (0.3 mm diameter) passed through the lumen at a resting tension of 1.0 g. For all tissues, one end was fixed to the bottom of the organ bath while the other was connected to a force-displacement transducer (TB-612T, Nihon Kohden) for the measurement of isometric force. The buffer solution contained modified Krebs-Henseleit solution with the following composition (mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. The buffer solution was maintained at 37 °C and oxygenated with 95 % O_2-5 % CO_2. After an equilibration period, the organ bath was replaced with Ca^{2+}-free 10^{-6} M nicardipine contained solution with the following composition (mM); NaCl 122.4, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 10.0 and EGTA 0.01. Fifteen min later, 1 mM ACh was added. All these functional studies were performed in the presence of 10^{-6} M indomethacin. This concentration of indomethacin had no effect on baseline tension and ACh-induced constriction of bronchial rings from both group (data not shown).

Drugs

The following drugs were used: 2,4-dinitrobenzene sulfonic acid sodium salt (Tokyo Kasei Co., Tokyo); acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo) and nicardipine, ethylene glycol-*bis*(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO), indomethacin (Sigma, St. Louis, USA). Indomethacin and nicardipine were dissolved in 10 mM Na_2CO_3 and DMSO, respectively, for a concentration of 10^{-4} M and administered in the organ bath in a final concentration of 10^{-6} M.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Dannett's *t*-test. Values of $P<0.05$ were taken to indicate a significant difference between groups of data.

Results

Our previous study revealed that the sensitization procedure used in the present study alone had no significant effect by itself on the ACh responsiveness of rat main bronchial preparations (**Experiment 2-2**). So in the present study, the bronchial responsiveness was compared between repeatedly antigen challenged group and nonsensitized normal control group. On the other hand, the concentration of nicardipine used completely blocked the high K^+ (10-90 mM)-induced bronchoconstriction in Ca^{2+} contained normal Krebs solution, indicating that voltage-dependent Ca^{2+} channels (VDCs) were completely blocked on this condition.

Fig. 6-1 shows the 1 mM ACh-induced constriction of rat bronchial rings in the presence of 10^{-6} M nicardipine under Ca^{2+}-free, 0.01 mM EGTA condition. In this condition, 1 mM ACh generated only a transient phasic constriction in all preparations used. The generated tension of bronchial rings from repeatedly antigen challenged rats (0.15 ± 0.04 g, n=5) was significantly greater than that from nonsensitized normal control group (0.02 ± 0.00 g, n=8; $P<0.05$).

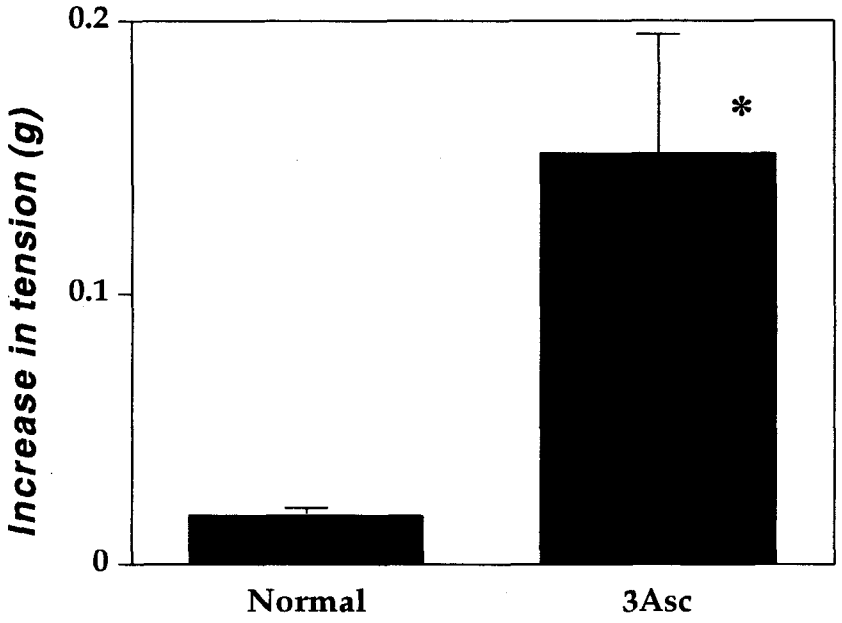


Figure 6-1

Effect of extracellular Ca^{2+} removal on the contractile response to acetylcholine (1 mM) of bronchial rings from nonsensitized normal control (Normal) and repeatedly antigen challenged (3Asc) rats. Bronchial rings were preincubated with nicardipine (10^{-6} M) in Ca^{2+}-free, 0.01 mM EGTA solution for 15 min. Values are means ± S.E. from 8 (Normal) and 5 (3Asc) experiments. *P<0.05.

Experiment 6-2: Effect of repeated antigenic challenge on Ca^{2+}-induced constriction of main bronchus pretreated with acetylcholine in Ca^{2+}-free medium.

Materials and methods

Sensitization and antigenic challenge

Animals were sensitized and received repeated antigenic challenge by the method described in **Experiment 6-1**.

In vitro determination of airway responses

The bronchial rings were prepared as described in **Experiment 6-1** and suspended in the organ bath. After an equilibration period, the organ bath was replaced with Ca^{2+}-free 10^{-6} M nicardipine contained solution with the following composition (mM); NaCl 122.4, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 10.0 and EGTA 0.01. Fifteen min later, 1 mM ACh was added and, after attainment of a plateau (almost baseline level) to the acetylcholine response, the concentration-response curve to Ca^{2+} (0.1-5.0 mM) was constructed cumulatively. A higher concentration of Ca^{2+} was successively added after attainment of a plateau response to the previous concentration. All these functional studies were performed in the presence of 10^{-6} M indomethacin. This concentration of indomethacin had no effect on baseline tension and ACh-induced constriction of bronchial rings from both group (data not shown).

Drugs

The following drugs were used: acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo) and nicardipine, ethylene glycol-*bis*(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO), indomethacin (Sigma, St. Louis, USA). Indomethacin and nicardipine were dissolved in 10 mM Na_2CO_3 and DMSO, respectively, for a concentration of 10^{-4} M and administered in the organ bath in a final concentration of 10^{-6} M.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Dannett's *t*-test. Values of $P<0.05$ were taken to indicate a significant difference between groups of data.

Results

Fig. 6-2 shows the concentration-response curves to Ca^{2+} of rat bronchial rings that were preincubated with 10^{-6} M nicardipine and 1 mM ACh under Ca^{2+}-free, 0.01 mM EGTA condition. The addition of Ca^{2+} induced a concentration-dependent constriction in either group. The contractile responses to Ca^{2+} of the acetylcholine stimulating muscles were markedly increased after repeated antigenic challenge.

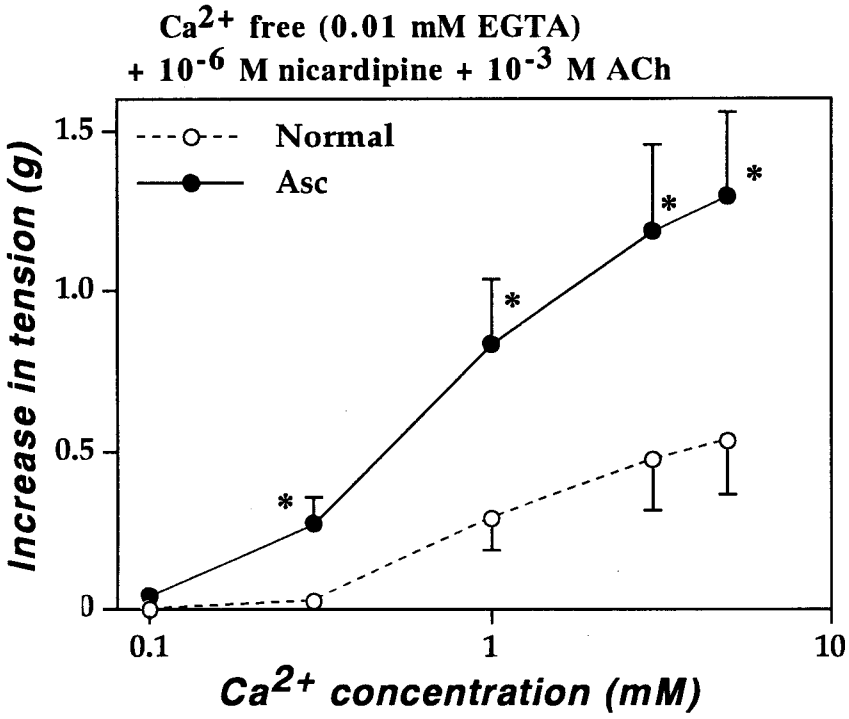


Figure 6-2

Cumulative concentration-response curves to Ca^{2+} of bronchial rings from nonsensitized normal control (Normal; O) and repeatedly antigen challenged (3Asc; ●) rats. Bronchial rings were preincubated with 10^{-6} M nicardipine and 1 mM acetylcholine in Ca^{2+}-free, 0.01 mM EGTA solution. Values are means ± S.E. from 8 (Normal) and 5 (3Asc) experiments. *P<0.05.

Experiment 6-3: Effect of repeated antigenic challenge on Ca^{2+}-induced constriction of main bronchus pretreated with high K^+ in Ca^{2+}-free medium.

Materials and methods

Sensitization and antigenic challenge

Animals were sensitized and received repeated antigenic challenge by the method described in **Experiment 6-1**.

In vitro determination of airway responses

The bronchial rings were prepared as described in **Experiment 6-1** and suspended in the organ bath. After an equilibration period, the organ bath was replaced with Ca^{2+}-free 10^{-6} M nicardipine contained solution with the following composition (mM); NaCl 122.4, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 10.0 and EGTA 0.01. Fifteen min later, bronchial smooth muscles were deporalized with 60 mM K^+ in the presence of 10^{-6} M atropine in the Ca^{2+}-free solution. Five min later, the concentration-response curve to Ca^{2+} (0.1-5.0 mM) was constructed cumulatively. A higher concentration of Ca^{2+} was successively added after attainment of a plateau response to the previous concentration. All these functional studies were performed in the presence of 10^{-6} M indomethacin. This concentration of indomethacin had no effect on baseline tension and high K^+-induced constriction of bronchial rings from both group (data not shown).

Drugs

The following drugs were used: atropine sulfate, ethylene glycol-*bis*(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and indomethacin (Sigma, St. Louis, USA). Indomethacin was dissolved in 10 mM Na_2CO_3 for a concentration of 10^{-4} M and administered in the organ bath in a final concentration of 10^{-6} M.

Statistical analyses

All the data are expressed as the mean with S.E. Statistical significance of difference was determined by Dannett's *t*-test. Values of $P<0.05$ were taken to indicate a significant difference between groups of data.

Results

Fig. 6-3 shows the concentration-response curves to Ca^{2+} of rat bronchial rings that were predepolarized with 60 mM K^+ under Ca^{2+}-free, 0.01 mM EGTA condition. The addition of Ca^{2+} induced a concentration-dependent constriction in either group. The responses to Ca^{2+} of the bronchial muscles depolarized with 60 mM K^+ (in the presence of 10^{-6} M atropine) were considered identical between groups.

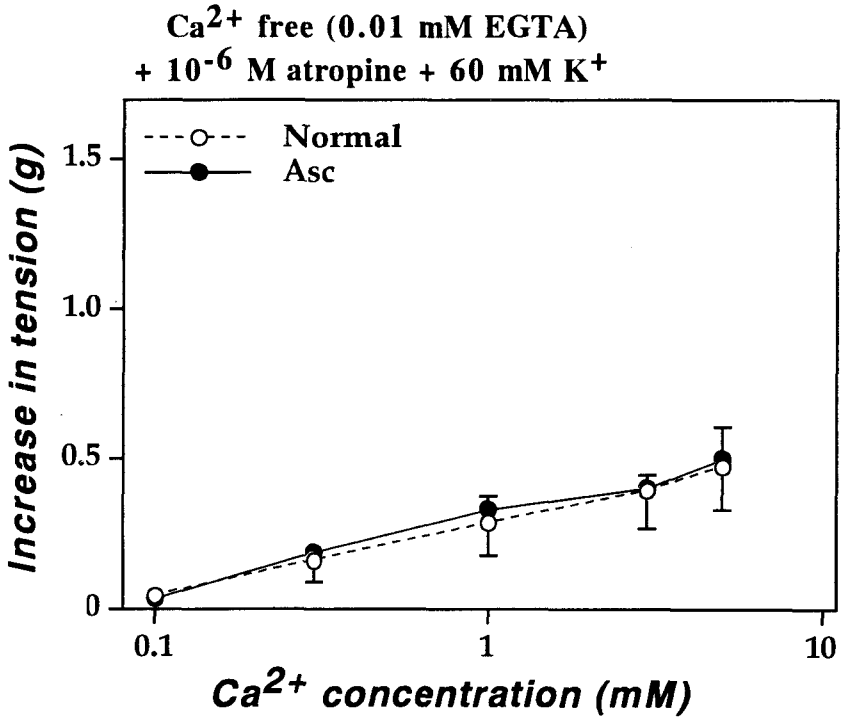


Figure 6-3

Cumulative concentration-response curves to Ca^{2+} of bronchial rings from nonsensitized normal control (Normal; O) and repeatedly antigen challenged (3Asc; ●) rats. Bronchial rings were deporalized with isotonic 60 mM K^+ in Ca^{2+}-free, 0.01 mM EGTA solution. Values are means ± S.E. from 8 (Normal) and 5 (3Asc) experiments.

Discussion

Airway smooth muscles are predominantly innervated by vagal efferent nerves, which release ACh when stimulated and subsequently activate muscarinic cholinergic receptors. The activation of muscarinic receptors existing on airway smooth muscle, that is mainly thought to be M_3 subtypes (Yang *et al.*, 1993), results in smooth muscle contraction by increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$): *i.e.* through Ca^{2+} release from sarcoplasmic reticulum and Ca^{2+} influx from voltage-dependent (VDCs) and receptor-operated Ca^{2+} channels (ROCs) (Barnes, 1990b; Montano *et al.*, 1993; Rodger & Pyne, 1992). Therefore, the increased ACh response of isolated bronchus from airway hyperresponsive rat may be attributable to enhanced Ca^{2+} mobilization at the AHR.

In the present study, no significant difference between groups in the contractile responses to cumulatively administered Ca^{2+} was observed in bronchial muscles that were predepolarized with 60 mM K^+ (Fig. 6-3), suggesting that no change in VDCs function occurs in bronchial smooth muscles of airway hyperresponsive rats. Taking this into consideration, it is unlikely that enhanced VDCs function is involved in the increased ACh responsiveness of the bronchus isolated from repeatedly antigen challenged rats. This might have relevance to the early finding that VDC-blocking drugs such as nifedipine have no effect for asthma therapy (Ferrari *et al.*, 1989). On the other hand, the Ca^{2+}-induced constriction of the muscles, that were preincubated with 1 mM ACh in the presence of 10^{-6} M nicardipine in Ca^{2+}-free 0.01 mM EGTA solution, was much greater in the AHR group than that in the normal group (Fig. 6-2). Furthermore, on this condition, the contractile response to ACh of bronchial rings from repeatedly antigen challenged rats was always significantly greater than that from control rats (Fig. 6-1). These findings strongly suggest that enhanced Ca^{2+} release from intracellular stores and/or influx from ROCs rather than from VDCs might be involved in the increased responsiveness to ACh of isolated bronchus from airway hyperresponsive rats.

It has been thought that activation of M_3 receptors promotes inositol 1,4,5-trisphosphate (IP_3) generation via activation of the effector enzyme, phospholipase C in airway smooth muscles. It has also been suggested that IP_3 stimulates the release of Ca^{2+} from sarcoplasmic reticulum, and inositol 1,3,4,5-tetrakisphosphate (IP_4; the metabolite of IP_3 by IP_3 kinase) may be responsible for opening putative ROCs (Luckhoff & Clapham, 1992; Rodger & Pyne, 1992), although the later process has not yet been demonstrated in muscle cells. Alternatively, it has been reported that activation of protein kinase C by diacylglycerol, another metabolite of phosphatidylinositol by phospholipase C, enhances Ca^{2+} sensitivity for smooth muscle contraction (Ozaki *et al.*, 1990: Sato *et al.*, 1992). Thus, although we do not yet understand whether increment of $[Ca^{2+}]_i$ level or enhancement of Ca^{2+} sensitivity occurs in this

phenomenon, we speculated that the resultant enhanced phosphatidylinositol turnover system in airway smooth muscles via the increased Ca^{2+} efficacy might be involved in the AHR in rats.

SUMMARY

Airway hyperresponsiveness (AHR) is a critical component in bronchial asthma, but the underlying mechanisms remain to be disclosed. Our first study in Chapter 1 was performed to establish a new good experimental animal model of AHR. Male Wistar rats actively sensitized with DNP-*Ascaris* antigen (DNP-Asc) were challenged by inhaling DNP-Asc 8 days after the first immunization (single antigenic challenge). Another sensitization group received 3 challenges every 48 hr (repeated antigenic challenge). The airway responsiveness to inhalations of acetylcholine (ACh) and neurokinin A (NKA) were determined under anesthesia. The airway microvascular leakage was also determined with Evans blue (E.B.). At 24 hr after the single antigen challenge, the *in vivo* airway responsiveness to ACh and NKA were slightly enhanced, and the E.B. exudation was increased in the main bronchus. In contrast, the *in vivo* airway responsiveness to ACh and NKA and the E.B. exudation in the main bronchus were much more markedly enhanced 24 hr after the repeated antigenic challenge. The isolated main bronchial strip from the challenged rats also acquired AHR towards ACh and serotonin. These findings suggest that repeated antigenic challenge causes a distinct AHR, both *in vivo* and *in vitro*, and that the airway inflammation may be closely related to pathogenesis of AHR.

In Chapter 2, strain differences in pathogenesis of antigen-induced AHR were investigated in Brown-Norway (BN) strain of rats, a high IgE responder, Long-Evans Cinnamon (LEC) strain of rats that has dysfunction of helper T cells, and Wistar strain of rats. Male BN, LEC and Wistar rats were actively sensitized with DNP-Asc together with *Bordetella pertussis* vaccine as an adjuvant. Five days later, the animals were boosted by DNP-Asc. At eight days after the first immunization, they were challenged by inhalation of the aerosolized DNP-Asc under unanesthesia. Three times repeated antigenic challenge was performed by inhaling DNP-Asc every 48 hr to sensitized rats. Twenty four hr after the last antigenic challenge, the *in vivo* airway responsiveness to cumulatively inhaled ACh was determined with modified Konzett-Rös sler method under immobilization and anesthesia. In Wistar rats, the airway responsiveness to inhaled ACh was significantly enhanced after repeated antigenic challenge, but not in BN and LEC rats. In *in vitro* studies, the response to cumulatively administered ACh of the isolated main bronchus from repeated antigenic challenged group was significantly augmented in Wistar rats, but not in BN and LEC rats. Only in Wistar rats, the wet/dry weight ratio of the left main bronchus, an index of airway tissue edema, was significantly increased after repeated challenge. The 48 hr PCA titer in BN rats was much higher than that in Wistar rats. These

results indicate that severe airway inflammation and AHR were induced in Wistar rats after repeated antigenic challenge, suggesting the relationship between AHR and airway inflammation, but not between AHR and serum IgE titer. Wistar rats may be most suitable for the model of antigen-induced AHR among the strains used.

In Chapter 3, the effects of ozagrel, a thromboxane A_2 (TXA_2) synthase inhibitor, and CV-3988, a platelet activating factor (PAF) antagonist, on the repeated antigenic challenge-induced AHR in rats were investigated. Rats were actively sensitized with DNP-*Ascaris* antigen and received 3 inhalations of antigen (challenges) or saline (sensitized control) every 48 hr. These animals were also pretreated with ozagrel (100 mg/kg, *p.o.*, 30 min before), CV-3988 (3 mg/kg, *i.v.*, 5 min before) or respective vehicle (water and saline, respectively) before each inhalation of antigen or saline. The *in vivo* airway responsiveness to cumulatively inhaled ACh (0.001-0.03 %, each for 3 min) was measured 24 hr after the last inhalation of antigen or saline under anesthesia. A marked AHR was observed after repeated antigenic challenge as compared with sensitized control group (5.5-9.5 times in potency). This AHR was significantly, but partly, attenuated by pretreatment with ozagrel although this treatment alone had no effect on the airway responsiveness to inhaled ACh in sensitized control animals. On the other hand, CV-3988 had no inhibitory effect on this AHR. These findings suggest that TXA_2, but not PAF, is one of the most important mediators participating in the pathogenesis of the antigen-induced AHR in rats.

In Chapter 4, the role of sensory neuropeptides in the AHR was investigated. First, the effect of the depletion of sensory neuropeptides by systemic capsaicin treatment on the AHR to ACh induced by repeated antigenic challenge to sensitized rats was studied. We secondly investigated whether the neutral endopeptidase (NEP) activity was altered at the antigen-induced AHR. Male Wistar rats were sensitized and repeatedly challenged with DNP-*Ascaris* antigen. Twenty-four hours after the last antigenic challenge, a marked AHR to inhaled ACh (0.001-0.03 %) was observed. This AHR was significantly attenuated by systemic capsaicin pretreatment prior to sensitization. On the other hand, in normal rats, the airway responsiveness to inhaled ACh was significantly increased by pretreatment with NEP inhibitor, phosphoramidon (3 mg/kg, i.v.), but the NEP inhibitor-induced effect was no more observed in the antigen-induced AHR rats. Furthermore, it was found that the airway NEP activity was significantly decreased at the antigen-induced AHR. These findings suggest that NEP hypoactivity and resultant increased sensory neuropeptides have an important role in the pathogenesis of antigen-induced AHR in rats.

In Chapter 5, the effects of sensory neuropeptides, NKA and substance P (SP), on the airway responsiveness to ACh were investigated both *in vivo* and *in vitro* by using normal

nonsensitized rats. The airway responsiveness to inhaled ACh was markedly and significantly increased after treatment with NKA (0.001 %) or SP (0.01 %) aerosol in the presence of the NEP inhibitor, phosphoramidon or thiorphan (1 and 3 mg/kg, i.v.). NKA had more potent effect than SP. Interestingly, the *i.v.* treatment with NEP inhibitor alone also induced AHR to inhaled ACh. This AHR was significantly attenuated by pretreatment with a nonselective NK-receptor antagonist, [D-Pro^2,D-Trp^{7,9}]-SP, systemic capsaicin or bilateral cervical vagotomy, indicating that decreased NEP activity results in accumulation of endogenous sensory neuropeptide(s) and enhancement of vagal reflex to cause the AHR. [^{3}H]QNB binding of the airway tissue membrane preparations of normal rats was not significantly affected after preincubation with phosphoramidon and/or NKA. On the other hand, the airway responsiveness to ACh of isolated left main bronchus was also increased after treatment with 10^{-6} M NKA, but not SP, together with 10^{-6} M phosphoramidon. This *in vitro* AHR to ACh induced by phosphoramidon plus NKA was significantly attenuated by pretreatment with 10^{-6} M tetrodotoxin. These findings suggest that over-accumulated sensory neuropeptides, especially NKA, may enhance the probability of transmitter release, probably via the NK_2-receptors, and that these enhanced probability of transmitter release might be involved in the AHR in rats.

In Chapter 6, the origin of Ca^{2+} contributing to the enhancement of ACh-induced bronchial smooth muscle constriction in AHR evoked by antigen challenge was investigated. Under Ca^{2+}-free (concomitant with 10^{-6} M nicardipine) condition, the contractile responses of bronchial rings to 1 mM ACh were significantly greater in airway hyperresponsive rats (0.15±0.04 g) than those in normal rats (0.02±0.00 g; $P<0.05$). The cumulatively administered Ca^{2+} induced a markedly greater bronchoconstriction in airway hyperresponsive rats in Ca^{2+}-free solution when muscles were pretreated with 1 mM ACh (in the presence of 10^{-6} M nicardipine) than in normal rats, whereas no significant difference in Ca^{2+} induced bronchoconstriction was observed between the two groups when muscles were pretreated with 60 mM K^+ (in the presence of 10^{-6} M atropine). These findings suggest that enhancement of availability of Ca^{2+} released from intracellular stores and/or influxed through receptor-operated Ca^{2+} channels in airway smooth muscles might be implicated in the AHR to ACh in rats.

CONCLUSION

In my present research, a good animal model of airway hyperresponsiveness (AHR) in allergic bronchial asthma was developed in rats. The mechanisms involved in the pathogenesis of AHR disclosed by using this AHR model are shown below:

1. A close relationship between the bronchial inflammation and developed AHR was demonstrated after repeated antigenic challenge to sensitized rats. This *in vivo* AHR is, at least in a part, ascribable to an increased bronchial smooth muscle responsiveness itself as shown in *in vitro* experiments.

2. Wistar strain of rats is the most suitable for the model of antigen-induced AHR and airway inflammation out of the strains tested: Wistar, Brown-Norway and Long-Evans Cinnamon.

3. Thromboxane A_2, but not platelet-activating factor, may be one of the most important mediators involved in the pathogenesis of antigen-induced AHR in rats.

4. The neutral endopeptidase hypoactivity in the airways and resultant increased endogenous sensory neuropeptides have an important role in the pathogenesis of antigen-induced AHR in rats.

5. As a role of sensory neuropeptides in the pathogenesis of AHR, activation of the NK_2-receptors on excitatory nerves by endogenous neurokinin A may enhance the transmitter release in the airways, which results in the enhanced bronchoconstriction (*i.e.*, AHR) in rats.

6. The enhancement of availability of Ca^{2+} released from intracellular stores and/or influxed through receptor-operated Ca^{2+} channels, not through voltage-dependent Ca^{2+} channels, in airway smooth muscles might be involved in the AHR to acetylcholine in rats.

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LIST OF PAPERS

This thesis is based on the following original publications:

1. Misawa, M. and Y. Chiba (1993) Repeated antigenic challenge-induced airway hyperresponsiveness and airway inflammation in actively sensitized rats. *Japan. J. Pharmacol.* **61**, 41-50.

2. Chiba, Y. and M. Misawa (1993) Strain differences in change in airway responsiveness after repeated antigenic challenge in three strains of rats. *Gen. Pharmacol.* **24**, 1265-1272.

3. Misawa, M. and Y. Chiba (1994) Inhibition of antigen-induced airway hyperresponsiveness in rats: effects of ozagrel (a thromboxane A_2 synthase inhibitor) and of CV-3988 (a platelet activating factor antagonist). *Res. Commun. Chem. Pathol. Pharmacol.* **84**, 341-349.

4. Chiba, Y. and M. Misawa (1994) Antigen-induced airway hyperresponsiveness is associated with airway tissue NEP hypoactivity in rats. *Life Sci.* **55**, 1919-1928.

5. Chiba, Y. and M. Misawa (1995) Inhibition of neutral endopeptidase increases airway responsiveness to acetylcholine in nonsensitized normal rats. *J. Appl. Physiol.* (in press).

6. Chiba, Y. and M. Misawa (1995) Alteration in Ca^{2+} availability involved in rat antigenically-induced airway hyperresponsiveness. *Eur. J. Pharmacol.* (in press).

REFERENCES

Abraham, W.M., J.S. Stevenson and R. Garrido (1988) A leukotriene and thromboxane inhibitor (SCH 37224) blocks antigen-induced immediate and late responses and airway hyperresponsiveness in allergic sheep. *J. Pharmacol. Exp. Ther.* **247**, 1004-1011.

Ackerman, S.J., D.A. Loegering, P. Vange, I. Olsson, J.B. Harley, A.S. Fauci and G.J. Gleich (1983) Distinctive cationic proteins of the eosinophilic granule: major basic protein, eosinophilic cationic protein and eosinophil-derived neurotoxin. *J. Immunol.* **131**, 2977-2982.

Agui, T., M. Oka, T. Yamada, T. Sakai, K. Izumi, Y. Ishida, K. Himeno and K. Matsumoto (1990) Maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in a mutant strain (LEC) of rat. *J. Exp. Med.* **172**, 1615-1624.

Aizawa, H., K.F. Chung, G.D. Leikauf, U. Ueki, R.A. Bethel, P.M. O'Byrne, T. Hirose and J.A. Nadel (1985) Significance of thromboxane generation in ozone-induced airway hyperresponsiveness in dogs. *J. Appl. Physiol.* **59**, 1918-1923.

Arnoux, B., D. Duval and J. Benveniste (1980) Release of platelet-activating factor (PAF-acether) from alveolar macrophages by the calcium ionophore A 23187 and phagocytosis. *Eur. J. Clin. Invest.* **10**, 437-441.

Arnoux, B., A. Denjean, C.P. Page, J. Morley and J. Benveniste (1983) Alveolar macrophages/paf-acether. A new association in the pathogenesis of human asthma. In *Platelet Activating Factor*, INSERM Symposium No. 23 (Eds. J. Benveniste and B. Arnoux), pp.335-341, Elsevier Science Publishers B.V., Amsterdam.

Bai, T.R. (1990) Abnormalities in airway smooth muscle in fatal asthma. *Am. Rev. Respir. Dis.* **141**, 552-557.

Bai, T.R. (1991) Abnormalities in airway smooth muscle in fatal asthma: a comparison between trachea and bronchus. *Am. Rev. Respir. Dis.* **143**, 441-443.

Baraniuk, J.N., J. Mak, M. LeTarte, R. Davis, C. Twort and P.J. Barnes (1991) Neutral endopeptidase mRNA expression in airways. *Am. Rev. Respir. Dis.* **143**, A40.

Barnes, P.J. (1986a) Neural control of human airways in health and disease. *Am. Rev. Respir. Dis.* **134**, 1289-1314.

Barnes, P.J. (1986b) Asthma as an axon reflex. *Lancet* **1**, 242-245.

Barnes, P.J. (1986c) Endogenous catecholamines and asthma. *J. Allergy Clin. Immunol.* **77**, 791-795.

Barnes, P.J. (1990a) Neural control of airway function: new perspectives. *Mol. Aspects Med.* **11**, 351-423.

Barnes, P.J. (1990b) Muscarinic receptors in airways: recent developments. *J. Appl. Physiol.* **68**, 1777-1785.

Barnes, P.J. (1991) Neuropeptides and asthma. *Am. Rev. Respir. Dis.* **143**, S28-S32.

Barnes, P.J. (1992a) Bradykinin as a mediator of asthma. *Thorax* **47**, 979-983.

Barnes, P.J. (1992b) Neuromodulation in the airways. *Physiol. Rev.* **72**, 699-729.

Barnes, P.J. and K.F. Chung (1987) PAF closely mimics pathology of asthma. *Trends Pharmacol. Sci.* **8**, 285-287.

Barnes, P.J., K.F. Chung and C.P. Page (1988) Inflammatory mediators and asthma. *Pharmacol. Rev.* **40**, 49-84.

Barnes, P.J., J. Baraniuk and M.G. Belvisi (1991a) Neuropeptides in the respiratory tract. *Am. Rev. Respir. Dis.* **144**, 1187-1198.

Barnes, P.J., J. Baraniuk and M.G. Belvisi (1991b) Neuropeptides in the respiratory tract. *Am. Rev. Respir. Dis.* **144**, 1391-1399.

Bateman, Jr., R.C., D. Jackson, C.A. Slaughter, S. Unnithan, Y.G. Chai, C. Moomaw and L.B. Hersh (1989) Identification of the active-site arginine in rat neutral endopeptidase 24.11 (enkephalinase) as arginine 102 and analysis of a glutamine 102 mutant. *J. Biol. Chem.* **264**, 6151-6157.

Bellofiore, S., G.U. Di Maria and J.G. Martin (1987) Change in upper and lower airway resistance after inhalation of antigen in sensitized rats. *Am. Rev. Respir. Dis.* **136**, 363-368.

Bellofiore, S. and J.G. Martin (1988) Antigen challenge of sensitized rats increases airway responsiveness to methacholine. *J. Appl. Physiol.* **65**, 1642-1646.

Black, J.L., L.M. Diment, L.A. Alouan, P.R.A. Johnson, C.L. Armour, T. Badgery-Parker and E. Burcher (1992) Tachykinin receptors in rabbit airways -characterization by functional, autoradiographic and binding studies. *Br. J. Pharmacol.* **107**, 429-436.

Boichot, E., V. Lagente, C. Carre, P. Waltmann, J.M. Mencia-Huerta and P. Braquet (1991) Bronchial hyperresponsiveness and cellular infiltration in the lung of guinea-pigs sensitized and challenged by aerosol. *Clin. Exp. Allergy* **21**, 67-76.

Borson, D.B (1991) Roles of neutral endopeptidase in airways. *Am. J. Physiol.* **260** (*Lung Cell. Mol. Physiol.* **4**), L212-L225.

Camussi, G., I. Pawlowski, C. Tetta, C. Roffinello, M. Alberton, J. Brentjens and G. Andres (1983) Acute lung inflammation induced in the rabbit by local instillation of 1-*O*-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphoryl-choline or of native platelet activating factor. *Am. J. Pathol.* **112**, 78-88.

Casale, T.B. and M.A. Kaliner (1984) Allergic reactions in the respiratory tract. In *Immunology of the Lung and Upper Respiratory Tract*, (Ed. J. Bienenstock), pp.236-344, McGraw-Hill, New York.

Cerrina, J., M.L.R. Ladurie, C. Labat, B. Raffestin, A. Bayol and C. Brink (1986) Comparison of human bronchial muscle responses to histamine in vivo with histamine and isoproterenol agonists in vitro. *Am. Rev. Respir. Dis.* **134**, 57-61.

Cheung, D., E.H. Bel, J. Den Hartigh, J.H. Dijkman and P.J. Sterk (1992) An effect of an inhaled neutral endopeptidase inhibitor, thiorphan, on airway responses to neurokinin A

in normal humans *in vivo*. *Am. Rev. Respir. Dis.* **145**, 1275-1280.

Chung, K.F. (1986) Role of inflammation in the hyperreactivity of the airway in asthma. *Thorax* **41**, 657-662.

Chung, K.F., H. Aizawa, A.B. Becker, O. Frick, W.M. Gold and J.A. Nadel (1986a) Inhibition of antigen-induced airway hyperresponsiveness by a thromboxane synthetase inhibitor (OKY-046) in allergic dogs. *Am. Rev. Respir. Dis.* **134**, 258-261.

Chung, K.F., H. Aizawa, G.D. Leikauf, I.F. Ueki, T.W. Evans and J.A. Nadel (1986b) Airway hyperresponsiveness induced by platelet-activating factor: Role of thromboxane generation. *J. Pharmacol. Exp. Ther.* **236**, 580-584.

Chung, K.F. and P.J. Barnes (1987) Bronchial hyperresponsiveness and inflammation in asthma. In *Asthma Reviews Vol. 1*, (editors), pp.25-41. Academic Press, London.

Chung, K.F., T.W. Evans, P.D. Graf and J.A. Nadel (1985) Modulation of cholinergic neurotransmission in canine airway by thromboxane mimetic U-46619. *Eur. J. Pharmacol.* **117**, 373-375.

Clark, P.O., D.J. Hanahan and R.N. Pinckard (1980) Physical and chemical properties of platelet-activating factor obtained from human neutrophils and monocytes and rabbit neutrophils and basophils. *Biochem. Biophys. Acta* **628**, 69-75.

Cockcroft, D.W., D.N. Killian, J.J.A. Mellon and F.E. Hargreave (1977) Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin. Allergy* **7**, 235-245.

Coleridge, J.C.G. and H.M. Coleridge (1984) Afferent vagal C fibre innervation of the lung and airways and its functional significance. *Rev. Physiol. Biochem. Pharmacol.* **99**, 1-110.

Connell, J.M.C. and A.G. Jardine (1990) *Atrial Natriuretic Factor*, A.D. Struthers (Ed), 181-194, Blackwell Scientific, Oxford.

Coyle, A.J., S.C. Urwin, C.P. Page, C. Touvay, B. Villian and P. Braquet (1988) The effect of the selective PAF antagonist BN 52021 on PAF and antigen-induced bronchial hyperreactivity and eosinophil accumulation. *Eur. J. Pharmacol.* **148**, 51-58.

Cuss, F.M., C.M. Dixon and P.J. Barnes (1986)Inhaled platelet activating factor causes bronchoconstriction and increased bronchial reactivity in man. *Am. Rev. Respir. Dis.* **133**, 212.

Daffonchio, L., A. Hernandez, L, Gallico and C. Omini (1990) Airway hyperreactivity induced by active cigarette smoke exposure in guinea-pigs: possible role of sensory neuropeptides. *Pulm. Pharmacol.* **3**, 161-166.

Danko, G., J.E. Sherwood, B. Grisson, W. Kreutner and R.W. Chapman (1988) Effect of the PAF antagonists, CV-3988 and L-652,731, on the pulmonary and hematological responses to guinea pig anaphylaxis. *Pharmacol. Res. Commun.* **20**, 785-798.

De Jongste, J.C., H. Mons, I.L. Bonta and K.F. Kerrebijn (1987) In vitro responses of airways from an asthmatic patient. *Eur. J. Respir. Dis.* **71**, 23-29.

De Monchy, J.G.R., H.F. Kauffman, P. Venge, G.H. Koeter, H.M. Jansen , H.J. Sluiter

and K. De Vris (1985) Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am. Rev. Respir. Dis.* **131**, 373-376.

Du, T., L.J. Xu, M. Lei, N.S. Wang, D.H. Eidelman, H. Ghezzo and J.G. Martin (1992) Morphometric changes during the early airway response to allergen challenge in the rat. *Am. Rev. Respir. Dis.* **146**, 1037-1041.

Dunnill, M.S. (1960) The pathology of asthma with special reference to changes in the bronchial mucosa. *J. Clin. Pathol.* **13**, 27-33.

Eidelman, D.H., S. Bellofiore and J.G. Martin (1988) Late airway responses to antigen challenge in sensitized inbred rats. *Am. Rev. Respir. Dis.* **137**, 1033-1037.

Elwood, W., J.O. Lötvall, P.J. Barnes and K.F. Chung (1991) Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitized Brown-Norway rats. *J. Allergy Clin. Immunol.* **88**, 951-960.

Evans, T.W., K.F. Chung, D.F. Rogers and P.J. Barnes (1987) Effect of platelet-activating factor on airway vascular permeability: possible mechanisms. *J. Appl. Physiol.* **63**, 479-484.

Fabbri, L.M., H. Aizawa, S.E. Alpert, E.H. Walters, P.M. O'Byrne, B.D. Gold, J.A. Nadel and M.J. Holtzman (1984) Airway hyperresponsiveness and changes in cell counts in bronchoalveolar lavage after ozone exposure in dogs. *Am. Rev. Respir. Dis.* **129**, 288-291.

Ferrari, M., M. Oliver, M. DeGasperi and A. Lechi (1989) Differential effects of nifedipine and diltiazem on methacholine-induced bronchospasm in allergic asthma. *Ann. Allergy* **63**, 196-200.

Filley, W.V., K.E. Holley, G.M. Kephart and G.J. Gleich (1982) Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* **2**, 11-15.

Flint, K.C., K.B.P. Leung, B.N. Hudspith, J. Brostoff, F.L. Pearce and N.M. Johnson (1985) Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *Br. Med. J.* **291**, 923-926.

Fox, A.J., P.J. Barnes, L. Urban and A. Dray (1993) An *in vitro* study of the properties of single vagal afferents innervating. *J. Physiol.* **469**, 21-35.

Frigas, E. and G.J. Gleich (1986) The eosinophil and the pathology of asthma. *J. Allergy Clin. Immunol.* **77**, 527-537.

Frew, A.J., R. Moqbel, M. Azzawi, A. Hartnell, J. Barkans, P.K. Jeffery, A.B. Kay, R.J. Scheper, J. Varley, M.K. Church and S.T. Holgate (1990) T lymphocytes and eosinophils in allergen-induced late-phase asthmatic reactions in the guinea pig. *Am. Rev. Respir. Dis.* **141**, 407-413.

Gibson, P.G., P.J. Manning, P.M. O'Byrne, A. Girgis-Gabardo, J. Dolovich, J.A. Denburg and F.E. Hargreave (1991) Allergen-induced asthmatic responses. *Am. Rev. Respir. Dis.* **143**, 331-335.

Gleich, G.J., E. Frigas, D.A. Loegering, D.L. Wassom and D. Steinmuller (1979) Cytotoxic properties of the eosinophil major basic protein. *J. Immunol.* **123**, 2925-2927.

Glynn, A.A. and L. Michaels (1960) Bronchial biopsy in chronic bronchitis and asthma. *Thorax* **15**, 142-153.

Godard, P., J. Chaintreuil, M. Damon, M. Coupe, O. Flandre, A. Crastes de Paulet and F.B. Michel (1982) Functional assessment of alveolar macrophages: comparison cells from asthmatics and normal subjects. *J. Allergy Clin. Immunol.* **70**, 88-93.

Goetzl, E.J., M.J. Phillips and W.M. Gold (1983) Stimulus specificity of the generation of leukotrienes by dog mastocytoma cells. *J. Exp. Med.* **158**, 731-737.

Goldie, R.G., D. Spina, P.J. Henry, K.M. Lulich and J.W. Patersson (1986) In vitro responsiveness of human asthmatic bronchus to carbachol, histamine, β-adrenoceptor agonists and theophylline. *Br. J. Clin. Pharmacol.* **22**, 669-676.

Gosset, P., A.B. Tonnel, M. Joseph, L. Prin, A. Mallart, J. Charan and A. Capron (1984) Secretion of a chemotactic factor for neutrophils and eosinophils by alveolar macrophages from asthmatic patients. *J. Allergy Clin. Immunol.* **74**, 827-834.

Grandel, K.E., E. Schwartz, D. Greene, M.L. Wardlow, R.N. Pinckard and R.S. Farr (1983) A platelet activating factor (PAF) extracted from acidified human plasma. *Fed. Proc.* **42**, 1026.

Guerzon, G.M., P.D. Pare, M.C. Michoud and J.C. Hogg (1979) The number and distribution of mast cells in monkey lungs. *Am. Rev. Respir. Dis.* **119**, 59-66.

Gundel, R.H., M.E. Gerritsen, G.J. Gleich and C.D. Wegner (1990) Repeated antigen inhalation results in a prolonged airway eosinophilia and airway hyperresponsiveness in primates. *J. Appl. Physiol.* **68**, 779-786.

Hall, A.K., P.J. Barnes, L.A. Meldrum and J. Maclagan (1989) Facilitation by tachykinins of neurotransmission in guinea-pig pulmonary parasympathetic nerves. *Br. J. Pharmacol.* **97**, 274-280.

Hargreave, F.E., G. Ryan, N.C. Thomson, P.M. O'Byrne, K. Latimer and E.F. Juniper (1981) Bronchial responsiveness to histamine or methacholine in asthma: measurement and clinical significance. *J. Allergy Clin. Immunol.* **68**, 347-355.

Havill, A.M., R.G. Van Valen and D.A. Handley (1990) Prevention of non-specific airway hyperreactivity after allergen challenge in guinea-pigs by the PAF receptor antagonist SDZ 64-412. *Br. J. Pharmacol.* **99**, 396-400.

Heffner, J.E., S.A. Shoemaker, E.M. Canham, M. Patel, I.F. McMurty, H.G. Morris and J.E. Repine (1983) Acetyl glyceryl ether phosphorylcholine-stimulated human platelet cause pulmonary hypertension and edema in isolated rabbit lungs. *J. Clin. Invest.* **71**, 351-357.

Hogg, J.C. (1981) Bronchial mucosal permeability and its relationship to airway hyperreactivity. *J. Allergy Clin. Immunol.* **67**, 421-426.

Holtzman, M.J., L.M. Fabbri, P.M. O'Byrne, B.D. Gold, H. Aizawa, E.H. Walters, S.E.

Alpert and J.A. Nadel (1984) Importance of airway inflammation for hyperresponsiveness induced by ozone. *Am. Rev. Respir. Dis.* **127**, 686-690.

Hooper, N.M., A.J. Kenny and A.J. Turner (1985a) The metabolism of neuropeptides: neurokinin A (substance K) is a substrate for endopeptidase 24.11 but not for peptidyl dipeptidase A (angiotensin-converting enzyme). *Biochem. J.* **231**, 257-261.

Hooper, N.M. and A.J. Turner (1985b) Neurokinin B is hydrolysed by synaptic membranes and by endopeptidase 24.11 ('enkephalinase') but not by angiotensin converting enzyme. *FEBS Lett.* **190**, 133-136.

Houston, J.C., S. De Nevasquez and J.R. Trounce (1953) A clinical and pathologic study of fatal cases of status asthmaticus. *Thorax* **8**, 207-213.

Humphrey, D.M., L.M. McManus, K. Satouchi, D.J. Hanahan and R.N. Pinckard (1982) Vasoactive properties of acetyl glyceryl ether phosphorylcholine and analogues. *Lab. Invest.* **46**, 422-427.

Hunninghake, G.W., I.I. Gallin and A.S. Fauci (1978) Immunologic reactivity of the lung. The in vivo and in vitro generation of a neutrophil chemotactic factor by alveolar macrophages. *Am. Rev. Respir. Dis.* **117**, 15-23.

Hunter, J.A., W.E. Finkbeiner, J.A. Nadel, E.J. Goetzl and M.J. Holtzman (1985) Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. *Proc. Natl. Acad. Sci. USA* **82**, 4633-4637.

Hutson, P.A., M.K. Church, T.P. Clay, P. Miller and S.T. Holgate (1988) Early and late-phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs. I. The association of disordered airway physiology to leukocyte infiltration. *Am. Rev. Respir. Dis.* **137**, 548-557.

Ishida, K., L.J. Kelly, R.J. Thomson, L.L. Beatti and R.R. Schellenberg (1989) Repeated antigen challenge induces airway hyperresponsiveness with tissue eosinophilia in guinea pigs. *J. Appl. Physiol.* **67**, 1133-1139.

Ishida, K., P.D. Pare, R.J. Thomson and R.R. Schellenberg (1990) Increased in vitro responses of tracheal smooth muscle from hyperresponsive guinea pigs. *J. Appl. Physiol.* **68**, 1316-1320.

Johnson, A.R., J. Ashton, W.W. Schulz and E.G. Erdos (1985) Neutral metalloendopeptidase in human lung tissue and cultured cells. *Am. Rev. Respir. Dis.* **132**, 564-568.

Joseph, M., A.B. Tonnel, G. Torpier and A. Chapron (1983) Involvement of immunoglobulin E in the secretory process of alveolar macrophages from asthmatic patients. *J. Cli. Invest.* **71**, 221-230.

Juniper, E.F., P.A. Frith and F.E. Hargreave (1981) Airway responsivenes to histamine and methacholine: relationship to minimum treatment to control symptoms asthma. *Thorax* **36**, 575-579.

Kleeberger, S.R., E.M. Wagner, G.K. Adams III, A.M. Dannenberg Jr. and E.W.

Spannhake (1985) Effect of repeated antigen exposure on antigen- and mediator-induced bronchospasm in sheep. *J. Appl. Physiol.* **59**, 1866-1873.

Komatsu, H., Y. Takehana, S. Kikuchi, M. Kojima, S. Hamano, H. Kusama, T. Okegawa and S. Ikeda (1990) Effect of a thromboxane A_2 synthetase inhibitor (OKY-046•HCl) on airway hyperresponsiveness in guinea pigs. *Eur. J. Pharmacol.* **184**, 87-95.

Konzett, H. and R. Rössler (1940) Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. Exp. Pathol. Pharmacol.* **135**, 71-76 (*in German*).

Kuo, H.-P., J.A.L. Rhode, K. Tokuyama, P.J. Barnes and D.F. Rogers (1990) Capsaicin and sensory neuropeptide stimulation of goblet cell secretion in guinea pig trachea. *J. Physiol.* **431**, 629-641.

Ladenius, A.R.C., J.W. Campbell, S. Sivaprasad, M.R. Suresh and D.F. Biggs (1991) Active immunization against substance P prevents the development of airway hyperresponsiveness in a guinea-pig model of asthma. *Am. Rev. Respir. Dis.* **143**, A614.

Laitinen, L.A., M. Heino, A. Laitinen, T. Kava and T. Haatela (1985) Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am. Rev. Respir. Dis.* **131**, 599-606.

Lammers, J.W.J., P.J. Barnes and K.F. Chung (1992) Non-adrenergic, non-cholinergic airway inhibitory nerves. *Eur. Respir. J.* **5**, 239-246.

Lammers, J.-W.J., P. Minette, M. McCusker, K.F. Chung and P.J. Barnes (1989) Capsaicin-induced bronchodilatation in mild asthmatic subjects: possible role of nonadrenergic inhibitory system. *J. Appl. Physiol.* **67**, 856-861.

Lang, Z., and C.G. Murlas (1991) HOCl exposure of a human airway epithelial cell line decreases its plasma membrane neutral endopeptidase. *Lung* **169**, 311-324.

Lee, T.-C., D.J. Lenihan, B. Malone, L.L. Roddy and S.I. Wasserman (1984) Increased biosynthesis of platelet-activating factor in activated human eosinophils. *J. Biol. Chem.* **259**, 5526-5530.

Lötvall, J.O., R.J. Lemen, K.P. Hui, P.J. Barnes and K.F. Chung (1990a) Airflow obstruction after substance P aerosol: contribution of airway and pulmonary edema. *J. Appl. Physiol.* **69**, 1473-1478.

Lötvall, J.O., B.-E. Skoogh, P.J. Barnes and K.F. Chung (1990b) Effects of aerosolized substance P on lung resistance in guinea-pigs: a comparison between inhibition of neutral endopeptidase and angiotensin-converting enzyme. *Br. J. Pharmacol.* **100**, 69-72.

Lötvall, J.O., K.P. Hui, C.-G. Löfdahl, P.J. Barnes and K.F. Chung (1991) Capsaicin pretreatment does not inhibit allergen-induced airway microvascular leakage in guinea pig. *Allergy* **46**, 105-108.

Luckhoff, A. and D.E. Clapham (1992) Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel. *Nature* **355**, 356-358.

Lundberg, J.M., T. Hokfelt, C.R. Martling, A. Saria and C. Cuello (1984) Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell Tissue Res.* **236**, 251.

Magnussen, H. and D. Nowak (1989) Roles of hyperresponsiveness and airway inflammation in bronchial asthma. *Respiration* **55**, 65-74.

Mak, J.C.W. and P.J. Barnes (1990) Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. *Am. Rev. Respir. Dis.* **141**, 1559-1568.

Mak, J.C.W., J.N. Baraniuk and P.J. Barnes (1992) Localization of muscarinic receptor subtype messenger RNAs in human lung. *Am. J. Respir. Cell Mol. Biol.* **7**, 344-348.

Marsh, W.R., C.G. Irvin, K.R. Murphy, B.L. Behrens and G.L. Larsen (1985) Increase in airway reactivity to histamine and inflammatory cells in bronchoalveolar lavage after the late asthmatic response in an animal model. *Am. Rev. Respir. Dis.* **131**, 875-879.

Martins, M.A., S.A. Shore and J.M. Drazen (1991) Release of tachykinins by histamine, methacholine, PAF, LTD_4, and substance P from guinea pig lungs. *Am. J. Physiol.* **261** (*Lung Cell. Mol. Physiol.* **5**), L449-L455.

Matsuse, T., R.J. Thomson, X.-R. Chen, H. Salari and R.R. Schellenberg (1991) Capsaicin inhibits airway hyperresponsiveness but not lipoxygenase activity or eosinophilia after repeated aerosolized antigen in guinea pigs. *Am. Rev. Respir. Dis.* **144**, 368-372.

McKay, K.O., J.L. Black and C.L. Armour (1992) Phosphoramidon potentiates the contractile response to endothelin-3, but not endothelin-1 in isolated airway tissue. *Br. J. Pharmacol.* **105**, 929-932.

Metzger, W.J., D. Zavala, H.B. Richerson, P. Moseley, P. Iwamota, M. Monick, K. Sjoerdsma and G.W. Hunninghake (1987) Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. Description of the model and local airway inflammation. *Am. Rev. Respir. Dis.* **135**, 433-440.

Misawa, M. and S. Iwamura (1990) Platelet-activating factor-induced rhinitis and involvement of PAF in allergic rhinitis in guinea pigs. *Japan. J. Pharmacol.* **54**, 217-226.

Misawa, M., K. Takenouchi, T. Abiru, Y. Yoshino and S. Yanaura (1987) Strain difference in an allergic asthma model in rats. *Japan. J. Pharmacol.* **45**, 63-68.

Misawa, M., Y. Kanai and Y. Chiba (1991) Effects of the new antiallergic drug epinastine and ketotifen on repeated antigen challenge-induced airway hyperresponsiveness in rats. *Arzneim.-Forsch.* **41**, 1277-1280.

Misawa, M. and T. Takata (1988) Effect of platelet-activating factor on rat airway. *Japan. J. Pharmacol.* **48**, 7-13.

Miura, M., M. Ichinose, K. Kimura (1992) Dysfunction of nonadrenergic noncholinergic inhibitory system after antigen inhalation in actively sensitized cat airways. *Am. Rev. Respir. Dis.* **145**, 70-74.

Miyoshi, R., S. Kito, K. Mizuno and H. Matsubayashi (1986) Is the effect of somatostatin on muscarinic receptors selective to M1 type? *Brain Res.* **377**, 348-350.

Montano, L.M., G.L. Jones, P.M. O'Byrne and E.E.Daniel (1993) Effect of ozone exposure in vivo on response of bronchial rings in vitro: role of intracellular Ca^{2+}. *J. Appl. Physiol.* **75**, 1315-1322.

Murlas, C.G. and J.H. Roum (1985) Sequencw of pathologic changes in the airway mucosa of guinea pigs during ozone-induced bronchial hyperreactivity. *Am. Rev. Respir. Dis.* **131**, 314-320.

Murlas, C.G., T.P. Murphy and Z. Lang (1990) HOCl causes airway substance P hyperresponsiveness and neutral endopeptidase hyporeactivity. *Am. J. Physiol.* **258** (*Lung Cell. Mol. Physiol.* **2**), L361-L368.

Murlas, C.G., Z. Lang, G.J. Williams and V. Chodimella (1992) Aerosolized neutral endopeptidase reverses ozone-induced airway hyperreactivity to substance P. *J. Appl. Physiol.* **72**, 1133-1141.

Nadel, J.A. and P.J. Barnes (1984) Autonomic regulation of the airways. *Ann. Rev. Med.* **35**, 451-467.

Nagai, H., F. Tsuji, N. Inagaki, K. Kitagaki, O. Fukutomi, A. Koda and M. Daikoku (1991) The effect of ONO-3708, a novel TXA_2 receptor antagonist, on U-46619-induced contraction of guinea pig and human tracheal strips in vitro and bronchoconstriction in guinea pigs in vivo. *Prostaglandins* **41**, 375-382.

Nakajima, H., I. Iwamoto, S. Tomoe, R. Matsumura, H. Tomioka, K. Takatsu and S. Yoshida (1992) $CD4^+$ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am. Rev. Respir. Dis.* **146**, 374-377.

Nambu, S., S. Murakata, T. Shiraji, N. Omawari, M. Sawada, T. Okegawa, A. Kawasaki and J. Ikeda (1990) OKY-046 inhibits anaphylactic bronchoconstriction and reduces histamine level in bronchoalveolar lavage fluid in sensitized guinea pigs. *Prostaglandins* **39**, 623-637.

Nichol, G.M., B. O'Connor, J.M. LeCompte, K.F. Chung and P.J. Barnes (1992) Effect of a neutral endopeptidase inhibitor on airway function in asthmatic subjects. *Eur. J. Clin. Pharmacol.* **42**, 495-498.

O'Byrne, P.M. (1986) Airway inflammation and airway hyperresponsiveness. *Chest* **90**, 575-577.

O'Byrne, P.M., H. Aizawa, R.A. Bethel, K.F. Chung, J.A. Nadel and M.J. Holtzman (1984) Prostaglandin $F_{2\alpha}$ increases responsiveness of pulmonary airways in dogs. *Prostaglandins* **28**, 537-543.

O'Byrne, P.M., G.D. Leikauf, H. Aizawa, R.A. Bethel, I.F. Ueki, M.J. Holtzman and J.A. Nadel (1985) Leukotriene B_4 induces airway hyperresponsiveness in dogs. *J. Appl. Physiol.* **59**, 1941-1946.

O'Donell, M.C., S.J. Ackerman, C.J. Gleich and L.L. Thomas (1981) Activation of basophil and mast cell histamine release by eosinophil granule major basic protein. *J. Exp. Med.* **157**, 1981-1991.

Okada, K., Y. Miyazaki, J. Takada, K. Matsumoto, T. Yamaki and M. Yano (1990) Conversion of big endothelin-1 by membrane-bound metalloendopeptidase in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.* **171**, 1192-1198.

Ollerenshaw, S.L., D. Jarvis, C.E. Sullivan and A.J. Woolcock (1991) Substance P immunoreactive nerves in airways from asthmatics and non-asthmatics. *Eur. Respir. J.* **4**, 673-682.

Ozaki, H., S.-C. Kwon, M. Tajimi and H. Karaki (1990) Changes in cytosolic Ca^{2+} and contraction induced by various stimulants and relaxants in canine tracheal smooth muscle. *Pflügers Arch.* **416**, 351-359.

Page, C.P. and J. Morley (1986) Evidence favouring PAF rather than leukotrienes in the pathogenesis of asthma. *Pharmacol. Res. Commun.* **18**, 217-235.

Pauwels, R., H. Bazin, B. Platteau and M. Van Der Streaten (1978) Effect of antigen dose on the secondary IgE response in BN rats. *Int. Arch. Allergy Appl. Immunol.* **57**, 472-476.

Pauwels, R., H. Bazin, B. Platteau and M. Van Der Streaten (1979) The influence of antigen dose on IgE production in different rat strains. *Immunology* **36**, 151-157.

Payan, D.G., J.D. Levine and E.J. Goetzl (1984) Modulation of immunity and hypersensitivity by sensory neuropeptides. *J. Immunol.* **132**, 1601-1604.

Peters, M.J., I.M. Adcock, C.M. Gelder, H. Shirasaki, M.G. Belvisi, M. Yacoub and P.J. Barnes (1992) NK_1-receptor gene expression is increased in asthmatic lung and reduced by corticosteroids. *Am. Rev. Respir. Dis.* **145**, A835.

Rand, M.J. (1992) Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clin. Exp. Pharmacol. Physiol.* **19**, 147-169.

Renzi, P.M., S. Sapienza, S. Waserman, T. Du, R. Olivenstein, N.S. Wang and J.G. Martin (1992) Effect of interleukin-2 on the airway responsiveness to antigen in the rat. *Am. Rev. Respir. Dis.* **146**, 163-169.

Roberts, J.A., D. Raeburn, I.W. Rodger and N.C. Thomson (1984) Comparison of in-vivo responsiveness and in-vitro smooth muscle sensitivity to methacholine. *Thorax* **39**, 837-843.

Robinson, C. and S.T. Holgate (1985) Mast cell-dependent inflammatory mediators and their putative role in bronchial asthma. *Clin. Sci.* **68**, 103-112.

Rodger, I.W. and N.J. Pyne (1992) Airway smooth muscle. In *Asthma: Basic Mechanisms and Clinical Management.* 2nd edn, (editors Barnes P.J., Roger I.W. and Thomson N.C.), pp.59-84. Academic Press, London.

Ronco, P., H. Pollard and M. Galceran (1988) Distribution of enkephalinase (membrane metalloendopeptidase, EC3.4.24.11) in rat organs. Detection using a monoclonal antibody. *Lab. Invest.* **58**, 210-217.

Sato, K., M. Hori, H. Ozaki, H. Takano, T. Tsuchiya, H. Sugi and H. Karaki (1992) Myosine phosphorylation-independent contraction induced by phorbol ester in vascular

smooth muscle. *J. Pharmacol. Exp. Ther.* **261**, 497-505.

Schellenberg, R.R. and A. Foster (1984) In vitro responses of human asthmatic airway and pulmonary vascular smooth muscle. *Int. Arch. Allergy Appl. Immunol.* **75**, 237-241.

Sekizawa, K., J. Tamaoki, P.D. Graf, C.B. Basbaum, D.B. Borson and J.A. Nadel (1987) Enkephalinase inhibitor potentiates mammalian tachykinin-induced contraction in ferret trachea. *J. Pharmacol. Exp. Ther.* **243**, 1211-1217.

Sekizawa, K., J. Tamaoki, J.A. Nadel and B. Borson (1987) Enkephalinase inhibitor potentiates substance P- and electrically induced contraction in ferret trachea. *J. Appl. Physiol.* **63**, 1401-1405.

Seltzer, J., B.G. Bigby, M. Stulbarg, M.J. Holtzman, J.A. Nadel, I.F. Ueki, G.D. Leikauf, E.J. Goetzl and H.A. Boushey (1986) O_3-Induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J. Appl. Physiol.* **60**, 1321-1326.

Shore, S.A., N.P. Stimler-Gerard, S.R. Coats and J.M. Drazen (1988) Substance P-induced bronchoconstriction in the guinea pig, enhancement by inhibitors of neutral metalloendopeptidase and angiotensin-converting enzyme. *Am. Rev. Respir. Dis.* **137**, 331-336.

Skidgel, R.A., S. Engelbrecht, A.R. Johnson and E.G. Erdos (1984) Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase. *Peptides* **5**, 769-776.

Solway, J. and A.R. Leff (1991) Sensory neuropeptides and airway function. *J. Appl. Physiol.* **71**, 2077-2087.

Spina, D., P.J. Rigby, J.W. Paterson and R.G. Goldie (1989) α-Adrenoceptor function and autoradiographic distribution in human asthmatic lung. *Br. J. Pharmacol.* **97**, 701-708.

Stretton, D., M.G. Belvisi and P.J. Barnes (1992) The effect of sensory nerve depletion on cholinergic neurotransmission in guinea pig airways. *J. Pharmacol. Exp. Ther.* **260**, 1073-1080.

Svensson, J., K. Strandberg, T. Tuvemo and M. Hamberg (1977) Thromboxane A_2: effects on airway and vascular smooth muscle. *Prostaglandins* **14**, 425.

Szarek, J.L. and J.N. Evans (1988) Pharmacologic responsiveness of rat parenchymal strips, bronchi, and bronchioles. *Exp. Lung Res.* **14**, 575-585.

Tada, T. and K. Okumura (1971) Regulation of homocytotropic antibody formation in the rat. I. Feed-back regulation by passively administered antibody. *J. Immunol.* **106**, 1002-1011.

Takahashi, N., Y. Aramaki and S. Tsuchiya (1990) Allergic rhinitis model with Brown Norway rat and evaluation of antiallergic drugs. *J. Pharmacobio-Dyn.* **13**, 414-420.

Takehana, Y., S. Kikuchi, K. Hara, S. Hamano, H. Komatsu, A. Ujiie, T. Okegawa and S. Ikeda (1990) Anti-allergic effects of OKY-046, a specific thromboxane A_2 synthetase inhibitor: Effects on type I allergic reactions. *Folia Pharmacol. Japon.* **95**, 121-130. (*in Japanese*)

Tanaka, D.T. and M.M. Grunstein (1986) Effect of substance P on neurally mediated contraction of rabbit airway smooth muscle. *J. Appl. Physiol.* **60**, 458-463.

Tanaka, D.T., R.E. Ando, G.L. Larsen and C.G. Irvin (1991) Cholinergic mechanisms involved with histamine hyperreactivity in immune rabbit airways challenged with ragweed antigen. *Am. Rev. Respir. Dis.* **144**, 70-75.

Thompson, P.J., J.M. Hanson, H. Bilani, M. Turner-Warwick and J. Morley (1984) Platelets, platelet activating factor and asthma. *Am. Rev. Respir. Dis.* **129**, 3.

Thompson, J.E., L.A. Scypinski, T. Gordon and D. Sheppard (1987) Tachykinins mediate the acute increase in airway responsiveness caused by toluene diisocyanate in guinea pigs. *Am. Rev. Respir. Dis.* **136**, 43-49.

Thompson, J.E. and D. Sheppard (1988) Phosphoramidon potentiates the increase in lung resistance mediated by tachykinins in guinea pigs. *Am. Rev. Respir. Dis.* **137**, 337-340.

Tousignant, C., C.-C. Chan, D. Guevremont, C. Brideau, J.J. Hale, M. MacCoss and I.W. Rodger (1993) NK_2 receptors mediate plasma extravasation in guinea-pig lower airways. *Br. J. Pharmacol.* **108**, 383-386.

Turk, J. R.L. Maas, A.R. Brash, L.J. Roberts and J.A. Oates (1982) Arachidonic acid 15-lipoxygenase products from human eosinophils. *J. Biol. Chem.* **257**, 7068-7076.

Turner, C.R., R.B. Stow, S.J. Hubbs, B.C. Gomes and J.C. Williams (1993) Acrolein increases airway sensitivity to substance P and decreases NEP activity in guinea pigs. *J. Appl. Physiol.* **74**, 1830-1839.

Udaka, K., Y. Tatenchi and H. Movat (1970) Simple method for quantitation of enhanced vascular permeability. *Proc. Soc. Exp. Biol. Med.* **133**, 1384-1387.

Ullrich, V. and R. Nusing (1990) Thromboxane synthase: from isolation to function. *Stroke* **21**(Suppl. IV), 134.

Umeno, E., J.A. Nadel, H.-T. Huang and D.M. McDonald (1989) Inhibition of neutral endopeptidase potentiates neurogenic inflammation in the rat trachea. *J. Appl. Physiol.* **66**, 2647-2652.

Umeno, E., J.A. Nadel and D.M. McDonald (1990) Neurogenic inflammation of the rat trachea: fate of neutrophils that adhere to venules. *J. Appl. Physiol.* **69**, 2131-2136.

Undem, B.J., A.C. Myers and D. Weinreich (1991) Antigen-induced modulation of autonomic and sensory neurons *in vitro*. *Int. Arch. Allergy Appl. Immunol.* **94**, 319-324.

Vincenc, K.S., J.L. Black, K. Yan, C.L. Armour, P.D. Donnelly and A.J. Woolcock (1983) Comparison of in vivo and in vitro responses to histamine in human airways. *Am. Rev. Respir. Dis.* **128**, 875-879.

Vornanen, M. and R. Tirri (1981) Cholinergic responses in different sections of rat airways. *Acta Physiol. Scand.* **113**, 177-182.

Wanner, A., W.M. Abraham, J.S. Douglas, J.M. Drazen, H.B. Richerson and J.S. Ram

(1990) NHLBI Workshop Summary. Models of airway hyperresponsiveness. *Am. Rev. Respir. Dis.* **141**, 253-257.

Wasserman, S.I. (1983) Mediators of immediate hypersensitivity. *J. Allergy Clin. Immunol.* **72**, 101-115.

Watanabe, A. and H. Hayashi (1990) Allergen-induced biphasic bronchoconstriction in rats. *Int. Arch. Allergy Appl. Immunol.* **93**, 26-34.

Wegner, C.D., R.H. Gundel, P. Reilly, N. Haynes, G.L. Letts and R. Rothlein (1990) Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. *Science* **247**, 456-459.

Weller, P.F., C.W. Lee, D.W. Foster, E.J. Corey, K.F. Austen and R.A. Lewis (1983) Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C_4. *Proc. Natl. Acad. Sci. USA* **80**, 7626-7630.

Widdicombe, J.G., J.-A. Karlsson and P.J. Barnes (1991) Cholinergic mechanisms in bronchial hyperreactivity and asthma. In *Asthma: Its Pathology and Treatment*, (eds. M.A. Kaliner, P.J. Barnes and C.G.A. Persson), pp.327-356, Marcel Dekker, New York.

Xu, L.J., D.H. Eidelman, J.H.T. Bates and J.G. Martin (1990) Late response of the upper airway of the rat to inhaled antigen. *J. Appl. Physiol.* **69**, 1360-1365.

Yamada, T., T. Natori, K. Izumi, T. Sakai, T. Agui and K. Matsumoto (1991) Inheritance of T helper immunodeficiency (*thid*) in LEC mutant rats. *Immunogenetics* **33**, 216-219.

Yang, C.M., Y.-L. Yo and Y.-Y. Wang (1993) Intracellular calcium in canine cultured tracheal smooth muscle cells is regulated by M_3 muscarinic receptors. *Br. J. Pharmacol.* **110**, 983-988.

Yonei, Y., P. Holzer and P.H. Guth (1990) Laparotomy-induced gastric protection against ethanol injury is mediated by capsaicin-sensitive sensory neurons. *Gastroenterology* **99**, 3-9.

Yoshida, M.C., R. Masuda, M. Sasaki, N. Takeuchi, H. Kobayashi, K. Dempo and M. Mori (1987) New mutation causing hereditary hepatitis in the laboratory rat. *J. Heredity* **78**, 361-365.